2015

Synthetic cannabinoids versus delta-9-tetrahydrocannabinol: abuse-related consequences of enhanced efficacy at the cannabinoid 1 receptor

Travis Grim
Virginia Commonwealth University

Synthetic cannabinoids versus delta-9-tetrahydrocannabinol: abuse-related consequences of enhanced efficacy at the cannabinoid 1 receptor

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

By

Travis William Grim

B.S. Virginia Polytechnic and State University

Director: Aron Lichtman, Ph.D.

Professor, Department of Pharmacology and Toxicology, Virginia Commonwealth University

Acknowledgements

I would like to thank my advisor, Aron Lichtman, for his ongoing support and belief in me. I came to him five and a half years ago with literally no lab experience; I could only offer enthusiasm and a willingness to learn. He took a chance and allowed me to get my first taste of biomedical science. He encouraged me to apply to the Biomedical Sciences Doctoral Portal program with only four days until the deadline, assuring me that it was possible. Perhaps I did not believe him at the time, but we persevered and submitted the application in time. Aron later offered me the opportunity to be his graduate student for which I was most grateful. The greatest gift he gave was the trust that I would figure things out for myself, and the encouragement to try my own ideas. I have learned a great deal under his guidance and I will continue down the path he helped me to find.

Steve Negus has been something of a second mentor, encouraging me to learn and explore all things related to pharmacology. We learned theory from afar in class; he taught me to breathe life into equations and theories and to mold and manipulate them myself. This dissertation very much bears his influence, and I am proud to have had the opportunity to interact with him.

Our department chair, Bill Dewey, supported me monetarily with his training grant, but also cultured the best pharmacology department one could hope for. To him, the people behind the science truly matter. I have the utmost respect for him.

I would like to thank my lab mates, past and present, for their contributions to my success. There have been many fruitful collaborations, and more importantly, many friends. I would also like to thank Richard Raabe and Jacy Jacobs for our mutual friendship that began in our first year and continues to this day.

I owe many thanks to my mother and my father who believed I could find success in everything that I attempted. My brother, Kramer, has been supportive as well, and I will miss the gym sessions when I move away.

Finally, I would like to thank Jennifer Cezar for her unwavering love and support through all of the ups and downs inherent to a graduate education. She has been there since my first year, and she will see me through to the end. I very much look forward to our adventures in Florida.

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[^{35}S]GTPγS	guanosine [^{35}S]5'-O-[gamma-thio]triphosphate
[3H]TMA	[^{3}H]trimethylammonium-D^{8}-tetrahydrocannabinol
11-OH-THC	11-hydroxy-Δ^{9}-tetrahydrocannabinol
2-AG	2-arachidonoylglycerol
AEA	arachidonoylethanolamide, anandamide
ANOVA	analysis of variance
BARR1	beta arrestin 1
BARR2	beta arrestin 2
cAMP	cyclic adenosine monophosphate
CB_1	cannabinoid receptor 1
CB_2	cannabinoid receptor 2
CNS	central nervous system
CPP	conditioned place preference
DEA	Drug Enforcement Administration
ERK	extracellular signal-regulated kinase
FAAH	fatty acid amide hydrolase
GABA	gamma-aminobutyric acid
GDP	guanosine biphosphate
GIRK	G-protein activated inward rectifying potassium
GPCR	G-protein coupled receptor
GRK	G-protein receptor kinase
GTP	guanosine triphosphate
hCB_1	human cannabinoid 1 receptor
HEK	human embryonic kidney
HET	heterozygous
ICSS	intracranial self-stimulation
KO	knockout
mAEA	methanandamide
MAGL	monoacylglycerol lipase
MPE	maximum possible effect
mRNA	messenger ribonucleic acid

NAcc	nucleus accumbens
OVX	ovarectemized
SA	self-administration
SAR	structure activity relationship
SC	synthetic cannabinoid
SEM	standard error of the mean
THC	Δ9-tetrahydrocannabinol
VTA	ventral tegmental area
WT	wild type

Abstract

SYNTHETIC CANNABINOIDS VERSUS DELTA-9-TETRAHYDROCANNABINOL: ABUSE-RELATED CONSEQUENCES OF ENHANCED EFFICACY AT THE CANNABINOID 1 RECEPTOR

By Travis Grim, B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2015

Major Director: Aron H. Lichtman, Ph.D., Department of Pharmacology and Toxicology

In the past ten years, synthetic cannabinoids (SC) have emerged as drugs of abuse. The first generation of these were research chemicals used to elucidate the existence and function of the cannabinoid receptor 1 (CB_1). Unlike Δ^9-tetrahydrocannabinol (THC), many SCs are associated with serious health complications and death. One way in which THC and SCs differ lies with their enhanced potency and efficacy at the CB_1 receptor. Accordingly, much lower concentrations of SCs are needed to elicit their THC-like effects, and many of these ligands are able to stimulate far more activity per receptor than THC. No current methods exist to measure efficacy at the CB_1 receptor *in vivo*, and the abuse-related properties of SC cannabinoids are not well explored. Here, we utilized CB_1 wild type (WT), heterozygous (HET), and knockout (KO) mice, which possess 100%, 50%, and 0% of normal CB_1 expression. By employing CB_1 ligands which differ in efficacy we have developed a method to explore the relationship between efficacy and the ability to produce cannabimimetic (catalepsy, hypothermia, and antinociception)

effects when CB_1 expression was reduced by half. Additionally, the intracranial self-stimulation procedure (ICSS) was utilized to investigate the effects of enhanced efficacy at CB_1 upon reward processes using representative SC CP55,940. As predicted, the potency shift between WT and HET mice inversely correlated with the efficacy of the test drug for both hypothermia and antinociception, but not catalepsy. This efficacy stratification was correlated with the agonist-stimulated [^{35}S]GTPγS binding assay, demonstrating this model as an effective tool to ascertain *in vivo* efficacy differences at CB_1. In ICSS, CP55,940 elicited only rate-decreasing effects acutely, although tolerance developed following repeated dosing. No evidence for spontaneous or rimonabant-precipitated withdrawal was observed. Together, these data indicate that highly efficacious cannabinoid ligands require few receptors to produce cannabimimetic effects, and that the model provides an effective means to quickly ascertain differences in efficacy. SCs continue to be a public health concern, and as they emerge their similarities to known cannabimimetic agents can be examined both in terms of efficacy and abuse-related effects.

Chapter 1: Introduction

1.1 Development and synthesis of cannabinoids as research tools

Written records and archeological evidence document that *Homo sapiens* have utilized the *Cannabis sativa* and *Cannabis indica* plants for recreational and medicinal purposes for at least 5000 years (Adrian, 2015). These uses of cannabinoids continue to the present day. Although inhalation of smoked cannabis or oral consumption produces well-described effects, including somnolescence (Belendiuk *et al*, 2015; Tart, 1970), euphoria as well as dysphoria, visual and auditory distortions of perception, elevated heart rate (Isbell *et al*, 1967; Weil *et al*, 1968), increased blood pressure, and impaired cognition (Waskow *et al*, 1970), the mechanisms by which cannabis (or marijuana) exerted its psychoactive effects were largely unknown until its chief primary psychoactive constituent, Δ^9-tetrahydrocannabinol (THC), was discovered. THC was first isolated in an impure form and its structure postulated more than seven decades ago (Wollner *et al*, 1942). In 1964, THC was again isolated from *Cannabis sativa* in a more pure preparation (Gaoni and Mechoulam, 1964). Subsequent work by Mechoulam showed THC extracted from marijuana and marijuana itself engendered similar psychoactive effects in humans (Mechoulam and Gaoni, 1967), and further examination of the composition of *Cannabis sativa* identified THC as a major component of marijuana (Mechoulam, 1970). The phytocannabinoid cannabidiol was also discovered at this time (Mechoulam *et al*, 1970; Mechoulam and Shvo, 1963), but unlike THC, it did not possess high affinity for CB_1 or CB2 (Showalter *et al*, 1996),

lacked cannabimimetic activity (Bloom *et al*, 1978; Chesher *et al*, 1973; Pertwee, 1972), and failed to substitute for THC's discriminative stimulus (Bueno *et al*, 1976; Järbe *et al*, 1977). Although these studies taken together implicated THC as the primary psychoactive ingredient of marijuana, they raised the following question: how was THC evoking its pharmacological effects?

Initial studies proposed various mechanisms to explain the pharmacological effects of THC such as plasma membrane perturbation or enzymatic interactions (Laurent and Roy, 1975; Roth and Williams, 1979). However, the concentrations of THC needed to achieve these effects were in the mid to upper micromolar range and far exceed the low or sub mg/kg doses sufficient to produce psychoactive effects in humans (Mechoulam and Gaoni, 1967). Promising mechanistic experiments conducted by Howlett and colleagues identified THC as a potent inhibitor of adenylyl cyclase. The findings that pharmacological blockade of a variety of receptors, including secretin, alpha adrenergic, serotonergic, and mu-opioid receptor antagonists (Howlett, 1984) did not prevent THC-induced inhibition of adenylyl cyclase suggest that a discrete cannabinoid receptor might exist and that it likely did not exert its pharmacological effects through the proposed disturbance of plasma membranes (Howlett and Fleming, 1984). Early radioligand displacement studies employing [^{3}H]trimethylammonium-Δ^8-tetrahydrocannabinol ([^{3}H]TMA) and unlabeled THC in rat brain homogenates strongly suggested discrete binding sites in a stereoselective manner, though potent displacement by cannabinoids with weak affinity for THC binding sites called into question the binding specificity of [^{3}H]TMA and precluded definitive identification of a discrete cannabinoid receptor (Nye *et al*, 1985). Fortunately, a series of potent cannabinoid ligands synthesized by Pfizer utilizing a bicyclic backbone structure provided highly useful tools for structure activity

relationship (SAR) and radioligand binding studies. A notable compound produced from these efforts was CP55,940, which was shown to behave similar to THC in inhibition of adenylyl cyclase (Howlett *et al*, 1988). This compound was subsequently radiolabeled with tritium and used to characterize the putative cannabinoid receptor(s) (Devane *et al*, 1988). [^{3}H]CP55,940 was utilized in autoradiography studies to characterize the distribution of cannabinoid receptor binding sites in the CNS (Herkenham *et al*, 1990). Proof of the existence of specific cannabinoid receptors culminated with the cloning of the CB_1 (Matsuda *et al*, 1990) and CB_2 (Munro *et al*, 1993) receptors, conclusively providing the sites of action by which cannabinoids exert their effects. The discovery of cannabinoid receptors would not have been possible without the synthesis of novel cannabinoids.

Other cannabinoids were developed for basic research as well as for potential therapeutic development. The classical cannabinoids possess the tricyclic structure characteristic of THC and include compounds such as HU series (Mechoulam *et al*, 1990). The alkylindole class of cannabinoids, including the JWH series (Aung *et al*, 2000) and the widely adopted WIN55,212-2 (Kuster *et al*, 1993), contained a great variety of backbone structures capable of binding and activating the CB_1 receptor. Similar to THC, many of these synthetic cannabinoids (SC) bound CB_1 and CB_2 (e.g. CP55,950; (Thomas *et al*, 1998), but the advent of the highly CB_1 selective antagonist SR141716A (or rimonabant) (Rinaldi-Carmona, 1994) revealed CB_1 in humans was the mechanism by which marijuana evoked its psychoactive effects (Huestis *et al*, 2001). Rimonabant also antagonized the *in vivo* subjective effects of CP55,940, WIN55,212-2, and THC in rats trained to discriminate THC from vehicle in the drug discrimination assay (Järbe and Henriksson, 1974; McMahon and Koek, 2007; Wiley *et al*, 1995) suggesting a similar mechanism of action between rodents and man. Finally, radiolabeled [^{3}H]SR141716A was used

to validate further that the majority of cannabinoid receptors in brain autoradiographs was CB_1 (Rinaldi-Carmona *et al*, 1996), cementing the CB_1 as the major cannabinoid receptor in the central nervous system. Cannabinoid ligands proved to be a promising molecules for numerous potential therapeutic indications, such as neuropathic pain, weight loss, and anxiolysis; thus, the push to synthesize and optimize new cannabinoids for these purposes continued. The cannabinoid field began to amass knowledge of hundreds of CB_1 ligands, and the SAR became very well understood for numerous scaffolds. Unwittingly, scientific publications that described the chemical synthesis of these drugs and their pharmacology set the stage for CB_1 agonists to be utilized for illicit purposes.

1.2 Pharmacological actions of marijuana, THC, and synthetic cannabinoids

Later efforts provided a more thorough investigation of the pharmacological effects of both marijuana and isolated THC in man and laboratory animals. Cardiovascular effects measured included elevated heart rate and increased blood pressure in human subjects (Isbell *et al*, 1967; Kochar and Hosko, 1973; Tashkin *et al*, 1973; Weil *et al*, 1968). The psychotropic effects in humans included subjective high (Weil *et al*, 1968), euphoria (Isbell *et al*, 1967), relaxation (Tart, 1970), memory impairments (Clark *et al*, 1970; Tinklenberg *et al*, 1970), disruption of cognitive tasks (Weil *et al*, 1968), and changes in temporal perception (Clark *et al*, 1970; Tinklenberg *et al*, 1970). Both anxiogenic (Zuardi *et al*, 1982) and anxiolytic (Fabre and McLendon, 1981) effects were also observed. In animal subjects, the subjective effects of cannabinoids were assessed utilizing the drug discrimination procedure, finding that generally, most cannabinoids substituted for THC and vice versa (Hruba *et al*, 2012; Järbe *et al*, 2014; McMahon, 2006; Wiley *et al*, 1995, 2013, 2014). *In vivo*, cannabinoids in rodents generally elicit hypolocomotion, hypothermia, catalepsy, and antinociception (Compton *et al*, 1992; Fan *et al*,

1994; Little *et al*, 1988a; McLaughlin *et al*, 2013), a constellation of effects generally referred to as the cannabinoid tetrad. Memory impairments (Ferrari *et al*, 1999; Heyser *et al*, 1993; Nakamura *et al*, 1991), anti-allodynic properties (Conti *et al*, 2002; Herzberg *et al*, 1997), and anxiety-related properties (Genn *et al*, 2004; Haller *et al*, 2002) were also commonly observed. Abuse-related behaviors represented a notable discrepancy between humans and laboratory animals.

1.3 Emergence of synthetic cannabinoids as drugs of abuse

With the growing scientific literature describing the synthesis of research compounds that bound CB_1 and produced cannabis-like effects, it should not be altogether surprising that many of these ligands eventually appeared as drugs of abuse. Indeed, John W. Huffman, the progenitor of the JWH cannabinoid series, was quoted in a Los Angeles Times interview saying "I always had a hunch that someday somebody would say: 'Hey, let's try smoking them.' And lo and behold, that's what happened" (Zucchino, 2011). Beginning in 2004, small packages of inert plant material imbued with unknown cannabimimetic agents labeled "not for human consumption" began to emerge (UNODC, 2014). Despite this disclaimer, these preparations with names such as "K2" and "Spice" were imbibed usually via smoking and soon precipitated unexpected health-related consequences. Ten cases of seizures were reported in Sweden in 2007 (EMCDDA, 2014), and soon after these incidents formal monitoring process of these abused preparations began. These events also spurred research to identify the chemical components that might be responsible for producing this alarming phenotype. The plant material itself was determined to be largely inert, serving primarily as a vehicle (EMCDDA, 2014). CP47,497 (Melvin et al., 1993), cannabicyclohexanol (Melvin et al., 1993), and JWH-018 (Wiley *et al*, 1998) were the first SCs positively identified in several different "Spice" products (Auwärter *et*

al, 2009). Their detection led to emergency scheduling of CP47,497, cannabicyclohexanol, JWH-018, JWH-073, and JWH-200 by the Drug Enforcement Administration (DEA) (The Drug Enforcement Administration, 2011), which in turn led the sellers to circumvent the law by using a new variety of SCs in their preparations. Subsequent emergency scheduling in 2013 of the SCs UR-144, XLR-11, APINACA, and AKB48 (The Drug Enforcement Administration, 2013), further actions in 2014 to schedule PB-22, 5F-PB-22, AB-FUBINACA, and ADB-PINACA (The Drug Enforcement Administration, 2014), and the more recent scheduling of AB-CHIMINACA, AB-PINACA, and THJ-2201 (The Drug Enforcement Administration, 2015) highlight the difficulty in keeping pace with the emergence of new SCs. Especially alarming is the emergence of compounds such as XLR-11, synthesized *de novo* with unknown pharmacology and toxicology (Center for Disease Control, 2013). This evidence suggests that newer SCs are likely produced by clandestine chemists with knowledge concerning structure activity relationships, given commonplace modifications, such as the addition of biosteric fluorine moieties that typically improve binding affinity at the CB_1 receptor (Banister *et al*, 2015). Each new cannabinoid structure also requires development of new methods of detection (Scheidweiler and Huestis, 2014), further complicating assessment of the risk to human health.

Numerous case reports point to potential health risks associated with SC use. Heart and kidney failure, respiratory depression, and seizures are repeatedly mentioned in case reports, as well as psychiatric indications, such as acute onset psychosis, anxiety, and cognitive impairment (Table 1). Alarmingly, a subset of synthetic cannabinoids has been linked to several deaths (Trecki *et al*, 2015). These reports are in marked contrast to THC, which has not been linked to a direct cause of death, despite its prolonged and high incidence of use. A large scale survey determined the risk of an emergency department visit is approximately 30 times higher for SCs

than for THC (Winstock *et al*, 2015), corroborating the trends seen in the plentiful case reports. The continued emergence of new SCs contributes to public health concerns of these drugs, but the underlying reasons for their higher health risk compared to THC remains poorly understood. Given the diversity in structures of abused cannabinoids, one explanation for these adverse events could be a non-CB_1 target which produces toxicity either through direct action of the parent compound or via metabolites. Alternatively, notable distinctions between THC and SCs are their abilities to bind and activate the CB_1 receptor. SCs are often more potent and more efficacious than THC (for review see Howlett, 2005). Thus, increases in the magnitude of CB_1 stimulation combined with high doses of SCs may potentially contribute to their toxic effects. In contrast, doses of THC obtained from marijuana consumption and its relatively low efficacy at the CB_1 may be insufficient to elicit these actions. Notable toxicological effects of high affinity, high efficacy SCs are consistent with of CB_1 expression in the relevant organs, such as the heart lungs, kidneys, and vast abundance in the central nervous system (CNS) (Galiègue *et al*, 1995), though a causal link between increased efficacy and the resulting deleterious health effects remains to be established. In order to evaluate the degree to which potency and/or efficacy at the CB_1 receptor contribute to the adverse effects of SCs, an efficient method of assessing these measures would be a helpful tool for both basic research and public health. The effects of SCs on CB_1 in the CNS represent an important aspect to study given the large number of psychiatric effects associated with their use.

Synthetic Cannabinoid	Health effects	Reference
"K2"	myocardial ischemia	Clark et al., 2015
PB-22	death	Gerostamoulos et al., 2015
5F-APINACA	driving impairment	Karinen et al., 2015
APINACA	driving impairment	
UR-144	driving impairment	
UR-144 degradant	driving impairment	
AM-2201	memory impairment, inappropriate giggling	Obafemi et al., 2015
AB-CHIMINACA	driving impairment, slurred speech, confusion	Peterson and Couper, 2015
AB-PINACA	driving impairment, slurred speech, confusion	
"K2"	withdrawal, seizures	Sampson et al., 2015
5F-PB-22	seizure	Schep et al., 2015
AM2233	Seizure	
BB-22	seizure	
JWH-122 (cyclohexylmethyl substituted)	seizure	
PB-22	seizure	
ADB-PINACA	delirium	Schwartz et al., 2015
XLR-11	death	Shanks et al., 2015
AB-PINACA	respiratory depression	Thornton et al., 2015
MDMD-CHMICA	sudden cardiac death	Westin et al., 2015
5F-PB-22	sudden death	Behonick et al., 2014
"K2"	acute cerebral ischemia, stroke	Bernson-Leung et al., 2014
XLR-11	acute kidney injury	Buser et al., 2014
AM-2201	schizophrenic symptoms	Celofiga et al., 2014
PB-22	seizure	Gugelmann et al., 2014
XLR-11	driving impairment, hypothermia, rigid muscle tone	Lemos, 2014
JWH-122	xerostomia, chest pain, tachycardia	Lonati et al., 2014
MAM-2201	xerostomia, chest pain, tachycardia	
"Black Diamond"	mutilation, amputation	Meijer et al., 2014
AM-2201	impaired driving, dizziness, somnolescence	Musshoff et al., 2014
JWH-018	impaired driving, dizziness, somnolescence	
JWH-019	impaired driving, dizziness, somnolescence	

JWH-122	impaired driving, dizziness, somnolescence	
JWH-210	impaired driving, dizziness, somnolescence	
JWH-307	impaired driving, dizziness, somnolescence	
MAM-2201	impaired driving, dizziness, somnolescence	
UR-144	impaired driving, dizziness, somnolescence	
"Spice/K2"	toxic hepatitis	Sheikh et al., 2014
XLR-11	acute cerebral ischemia	Takematsu et al., 2014
AM-2201	driving impairment	Tuv et al., 2014
JWH-018	driving impairment	
JWH-081	driving impairment	
JWH-122	driving impairment	
JWH-250	driving impairment	
RSC-4	driving impairment	
AM-2201	diffuse pulmonary infiltrates	Alhadi et al., 2013
JWH-122	diffuse pulmonary infiltrates	
JWH-210	diffuse pulmonary infiltrates	
"Spice"	oliguric acute kidney injury	Bhanushali et al., 2013
JWH-018	acute cerebral ischemia, stroke	Freeman et al., 2013
JWH-018	mild agitation, laugh attacks, panic attacks, vomiting, myoclonic jerking	Hermanns-Clausen et al., 2013
JWH-018	mydriasis, aniscoria, retrograde amnesia, somnolescence	
JWH-018	mydriasis, mild tachycardia, hypokalemia, leukocytosis	
JWH-018	seizures, difficulty breathing	
JWH-073	mild agitation, laugh attacks, panic attacks, vomiting, myoclonic jerking	
JWH-081	mild agitation, laugh attacks, panic attacks, vomiting, myoclonic jerking	
JWH-122	mydriasis, aniscoria, retrograde amnesia, somnolescence	
JWH-122	mydriasis, mild tachycardia, hypokalemia, leukocytosis	
JWH-122	seizures, difficulty breathing	
JWH-210	seizures, difficulty breathing	

UR-144	mydriasis, aniscoria, retrograde amnesia, somnolescence	
AM-2201	cannabinoid hyperemesis	Hopkins and Gilchrist, 2013
AM-694	cannabinoid hyperemesis	
JWH-0122	cannabinoid hyperemesis	
JWH-018	cannabinoid hyperemesis	
JWH-073	cannabinoid hyperemesis	
AM-2201	seizures	McQuade et al., 2013
"Mr. Nice Guy"	withdrawal not alleviated by THC, anxiety, cramps, loss of appetite	Nacca et al., 2013
THC	withdrawal not alleviated by THC, anxiety, cramps, loss of appetite	
AM2201	psychiatric complications, death, mutilation	Patton et al., 2013
JWH-073	psychiatric complications, death, mutilation	
XLR-11	acute kidney injury	Thornton et al., 2013
AM-2201	driving impairment	Yeakel and Logan, 2013
JWH-081	driving impairment	
JWH-122	driving impairment	
JWH-210	driving impairment	
JWH-250	driving impairment	
"K2"	catatonic, non-responsive to verbal or painful stimuli	Cohen et al., 2012
"Spice"	aggression, tachycardia	
"K2"	emesis, tachycardia	Faircloth et al., 2012
"K2"	respiratory depression	Jinwala and Gupta, 2012
"Spice"	psychosis	Peglow et al., 2012
"K2"	suicidal ideation	Thomas et al., 2012
JWH-018	respiratory depression, tachycardia, fever	Tofighi and Lee, 2012
JWH-018	seizure, tachyarrhythmia	Lapoint et al., 2011
"K2"	myocardial infarction	Mir et al., 2011
JWH-018	anxiety	Schneir et al., 2011
JWH-073	anxiety	
JWH-018	tachycardia, unresponsiveness	Simmons et al., 2011
JWH-073	tachycardia, unresponsiveness	
"Spice Gold"	anxiety, nocturnal nightmares, sweating, nausea, tremors, headache	Zimmermann et al., 2009

Table 1. A summary of case reports detailing the health effects linked to specific SCs detected in abused preparations. Many earlier SCs were diverted research chemicals, while later generations of "Spice" and "K2" preparations possessed SCs with novel structures and unknown pharmacology.

1.4 Structure activity relationships of synthetic cannabinoids.

Cannabinoid SAR studies used THC as a molecular scaffold to explore the effects of structural modifications in producing cannabimimetic activity. As depicted in Figure 1, four primary pharmacophores on the molecule were targeted for structural alterations: 1) alkyl chain length at C-3, 2) the hydroxyl at the C-1 position, 3) the methyl at the C-9 position, and 4) the "B" ring at the C-6 position. The earliest SAR studies explored the alkyl chain length and methyl substitutions at the first position on the alkyl chain in producing static ataxia in dogs (Adams *et al*, 1948a, 1948b), and found that 1′,2′-dimethyl-heptyl chains were the most potent. Martin and colleagues demonstrated that the alkyl chain length was an important determinant of *in vivo* potency (Martin *et al*, 1999). Structural modifications of the dimethylheptylpyran backbone elucidated the SAR of these highly potent cannabinoids (Razdan and Dalzel, 1976). Later work by Mechoulam and would explored the stereospecificity of the dimethyl additions at the first position of the alkyl chain, leading to the synthesis of HU-210, a highly potent synthetic cannabinoid approximately 85 times more potent than Δ^1-tetrahydrocannabinol to produce subjective effects in rats (Mechoulam *et al*, 1988). Modifications to the hydroxyl at the C-1 position revealed a mechanism to convey CB_2 selectivity via methylation of the hydroxyl (Pertwee *et al*, 2000; Zitko *et al*, 1972) as measured in radioligand binding studies. The C-11 methyl group was determined to be nonessential for binding, but modifications, such as ketone (nabilone) (Archer *et al*, 1977) or dimethyl hydroxyl (HU-210) (Mechoulam *et al*, 1988) groups enhanced CB_1 receptor binding affinity. Representative classical, tricyclic cannabinoids (Figure 2) demonstrated modifications at the aforementioned positions alongside THC. The aforementioned structures utilized the tricyclic structure of cannabinoids, but work by chemists from Pfizer utilized the bicyclic CP47,497 as the backbone structure to reveal the "B" ring as

non-essential (Figure 3), while the other three pharmacophores continued to exert their predicted effects on binding affinity to cannabinoid receptors (Melvin and Johnson, 1987; Melvin *et al*, 1993a).

The aminoalkylindole WIN55,212-2 was initially developed as a structural derivative of pravadilone, a non-acidic, potent inhibitor of cyclooxygenase enzymes. Surprisingly, WIN55,212-2 and pravadilone inhibited electrically-evoked mouse vas deferens contractions, inhibited adenylyl cyclase, and possessed greater than expected antinociceptive properties (Bell *et al*, 1991). WIN55,212-2 was later discovered to bind to cannabinoid receptors with nanomolar affinity, which accounted for its unexpected *in vivo* and *in vitro* cannabimimetic activity, and it failed to appreciably inhibit prostaglandin synthesis (D'Ambra *et al*, 1992). Ensuing studies investigated the SAR of this new class of cannabinoid compounds. Much of the SAR work in this series of compounds was performed in collaboration with John W. Huffman, who helped established the amino portion of the aminoalkyl side chain could be eschewed for an alkyl chain, thus resembling more closely the classical and non-classical cannabinoids (Wiley *et al*, 1998). Important contributions were also made by Alex Makriyannis, who demonstrated common pharmacological moieties between classical cannabinoids and the seemingly structurally dissimilar aminoalkylindoles (Xie *et al*, 1995). The benzene ring was also discovered to be nonessential (Figure 4), and from these modifications the JWH series was born including the eventually abused JWH-018 and JWH-073 (Huffman, 1999; Huffman *et al*, 1994).

Other cannabinoid SAR studies searched for CB_2-selective indoles and pyrroles for therapeutic purposes, though many of these compounds retained appreciable affinity for the CB_1 receptor (Frost *et al*, 2010). Once emergency scheduling events began (The Drug Enforcement Administration, 2011), a rapid increase in clandestine synthesis of structurally unique

cannabinoids began, resulting in myriad structures designed to maintain cannabimimetic effects while evading legal restrictions and established assays of detection, accomplished by utilizing small substitutions such as fluorination (Banister *et al*, 2015; Gatch and Forster, 2014). Adamantylindoles, such as APICA and APINACA, became increasingly found in preparations and were subsequently scheduled (Figure 5) (The Drug Enforcement Administration, 2013, 2014). Unlike the older generation of SCs, new SCs were synthesized to evade illegality and generally lack the preclinical testing, which resulted in abused compounds that possessed unknown pharmacodynamics, pharmacokinetics, and toxicology. To date, the relationship between enhanced efficacy and potency at the CB_1 receptor and the health risks remains unexplored, though SCs generally possess much higher efficacy and affinity at the CB_1 receptor than THC.

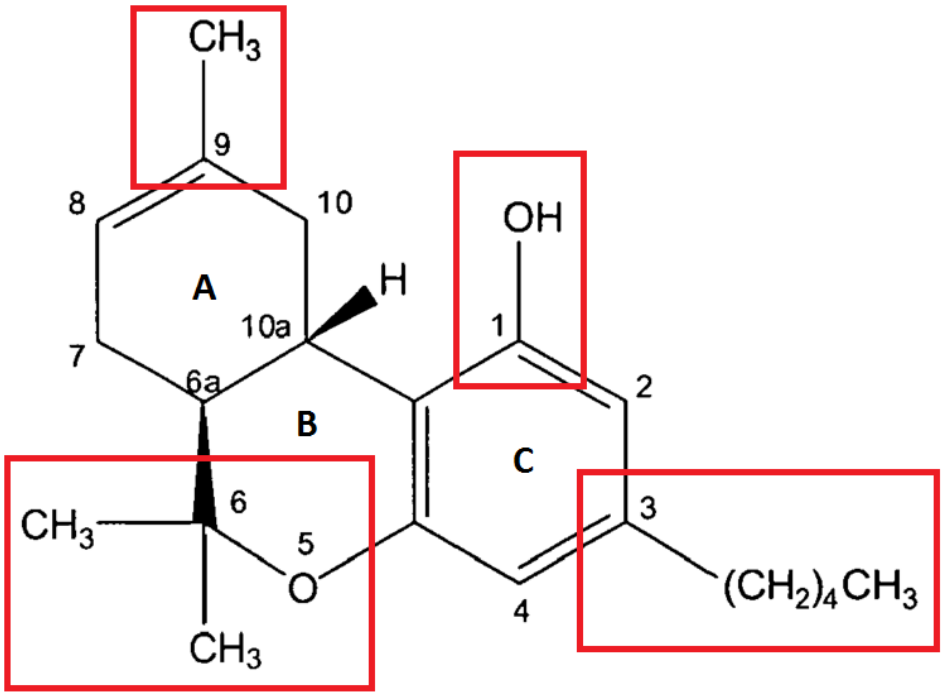

Figure 1. The pharmacophores of THC were established via SAR studies. The alkyl chain at C-3 was determined to be an important determinant of both binding affinity and efficacy, with an optimal chain length of 6-7 carbons. An addition of a dimethyl group at the first carbon of the chain also improved binding affinity. Substitution of the C-1 or C-9 pharmacophores could drastically change binding affinity at the CB_1 receptor, while methylation of the C-1 hydroxyl could enhance selectivity for the CB_2 receptor. The B ring, specifically the oxygen at position 5 and the carbon at position 6, was determined to be nonessential via SAR work utilizing Pfizer's nonclassical, bicyclic cannabinoid series.

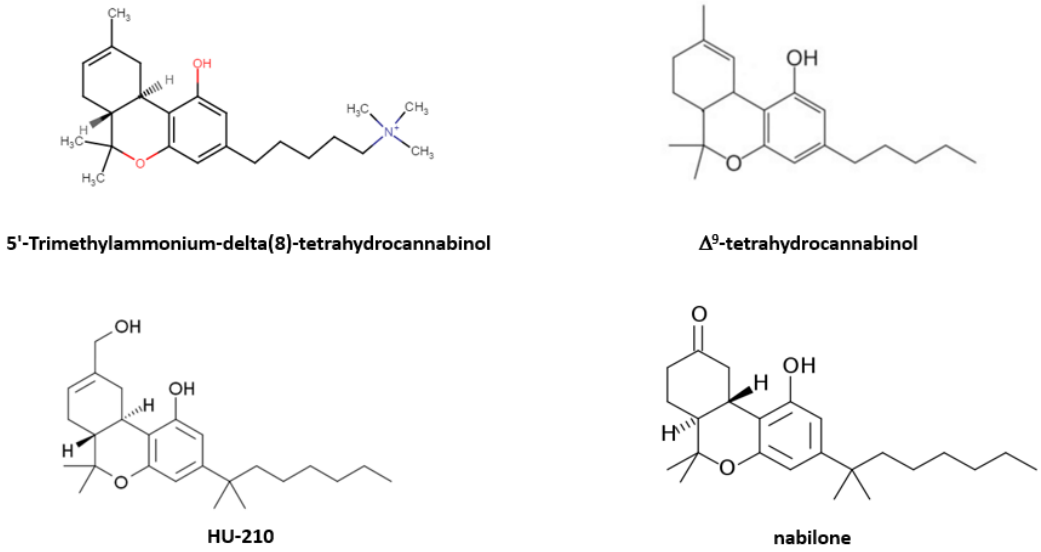

Figure 2. Classical cannabinoids, based upon the structure of THC, were instrumental in discovering the CB_1 receptor. Researchers identified discrete cannabinoid binding in rat brains utilizing the THC analogue 5´-trimethylammonium-Δ^8-tetrahydrocannabinol. HU-210, synthesized by Mechoulam and colleagues, allowed investigation of the stereospecificity of cannabinoids, strongly suggesting the presence of a discrete cannabinoid receptor. Likewise, nabilone represents a series of structural modifications to improve binding affinity of THC, notably the dimethyl substitution at the first carbon of the alkyl chain at C-3 and the ketone at C-9.

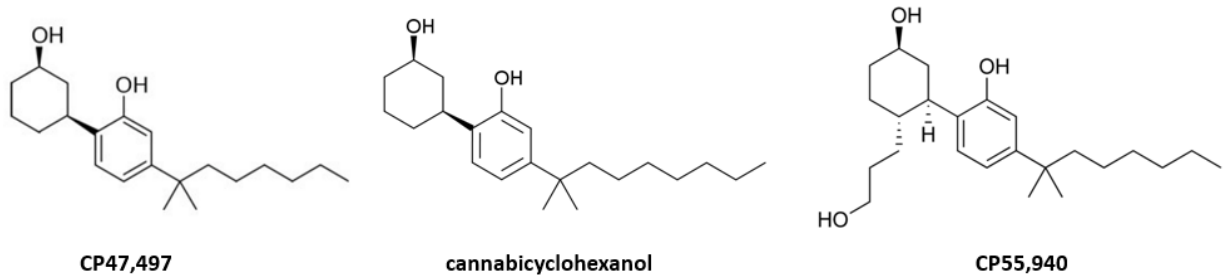

Figure 3. Nonclassical cannabinoids developed by Pfizer were instrumental in demonstrating the B ring of classical cannabinoids as nonessential for cannabinoid receptor binding and cannabimimetic activity. CP47,497 and its C-8 homologue cannabicyclohexanol were among the first synthetic cannabinoids detected in abused "Spice" and "K2" formulations. CP55,940 emerged as an important research tool to explore cannabinoid pharmacology *in vitro* and *in vivo*, and contributed to the discovery of cannabinoid receptor distribution in the CNS via autoradiography studies.

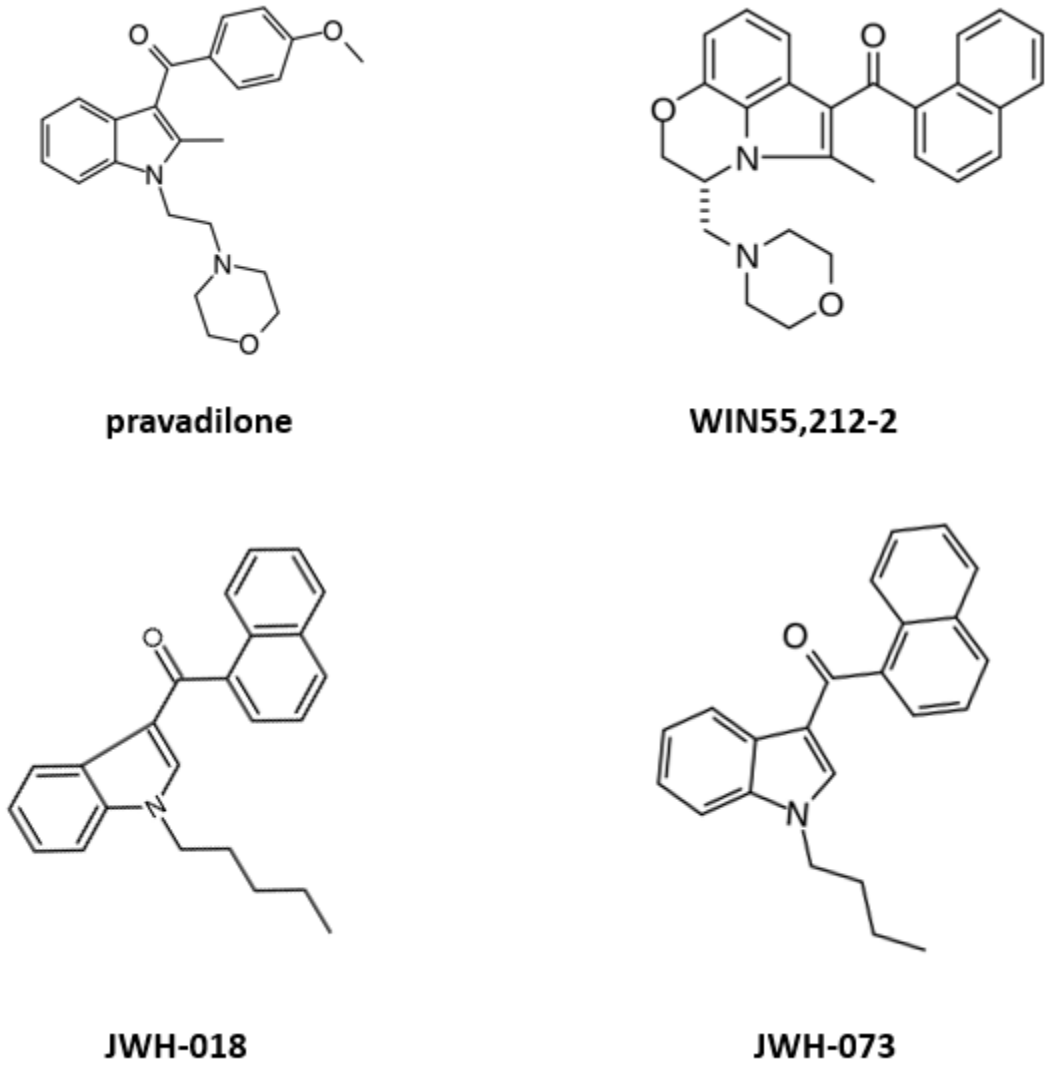

Figure 4. The serendipitous discovery of the indole backbone based upon the structure of the non-steriodal anti-inflammatory drug pravadilone led to the synthesis of many cannanabinoids. WIN55,212-2 was among the first of the cannabimimetic indoles and became popular as a research tool in part due to its high efficacy at the CB_1 receptor. Later SAR experiments by John W. Huffman determined the benzene ring of the indole to be nonessential for cannabimimetic activity as demonstrated by JWH-018 and JWH-073. Along with many other cannabinoids from the JWH series, JWH-018 and JWH-073 were eventually detected in abused preparations.

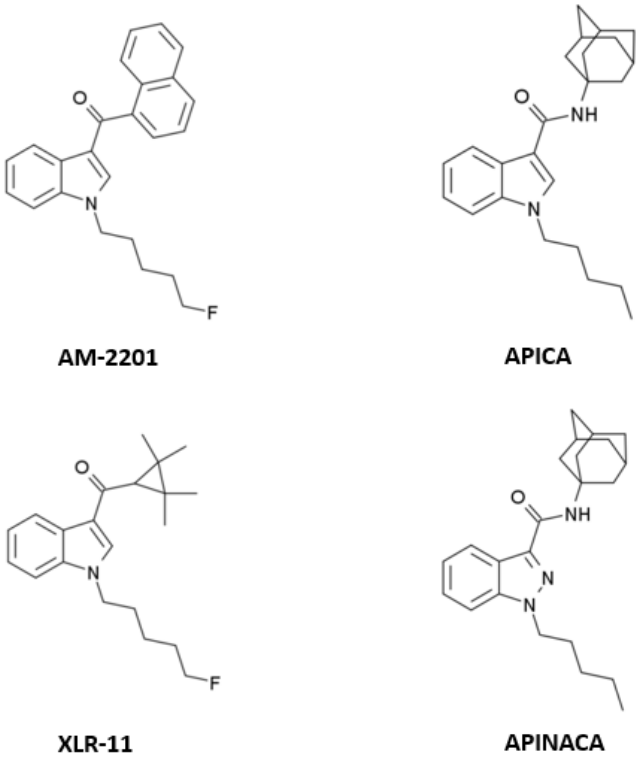

Figure 5. Following the scheduling of many of the first wave of abused synthetic cannabinoids, a huge variety of structures were detected via toxicological screens following emergency department visits. AM-2201 and XLR-11 represented fluorinated analogues of the scheduled JWH-018 and UR-144, respectively, and reveal how small structural modifications were made to sidestep scheduling of their parent compounds. APICA and APINACA, similar to the JWH series, contained the indole backbone, but the addition of the adamantyl moiety instead of the napthyl exhibit the large variety of cannabinoid structures present in the modern abused preparations.

1.5 Determination of efficacy utilizing receptor theory

Modern pharmacology was born of physiologists attempting to empirically determine the underpinnings of drug interactions with the tissues they acted upon. The concept of the receptor was a theoretical "receptive substance" for a given compound as described by Langley (Langley, 1905). Clark later expanded this treatment by Langley to incorporate the law of mass action, defining explicitly the relationships between the amount of ligand present and the amount of available receptors. Briefly, the ligand-receptor complexes associate and dissociate at a rate proportional to the number of complexes (Stephenson, 1956). Thus, at equilibrium (or occupation of half of the available receptors) one may determine the affinity of the ligand. Clark applied this concept to relate the number of ligand-receptor complexes to the response of the tissue, generating the familiar hyperbolic dose-response relationship (Clark, 1926, 1927). However, Clark also assumed the fractional occupancy was proportional to the tissue response, which later experiments would demonstrate to be a false assumption. Subsequently, Stephenson showed that compounds varied in their intrinsic activity, or efficacy, at a given target (Stephenson, 1956). This conceptual frame work led to the definition of a partial agonist as "low efficacy compounds possess properties intermediate to agonists and antagonists." Much of this work culminated in the two-state model (Black and Leff, 1983), which related the concepts of fractional occupancy, receptor number, and efficacy into a single equation. From this derivation also came the postulate that to differentiate between the efficacies of ligands, receptors number must be reduced to deplete spare receptors, such that rightward and downward shifts in dose-response relationships might be revealed.

In vitro methods to ascertain efficacy at the CB_1 receptor have largely focused on agonist-stimulated guanosine [^{35}S]5'-O-[gamma-thio]triphosphate ([^{35}S]GTPγS) binding. This assay enabled measuring efficacy at the first step of the canonical G-protein-adenylyl cyclase-protein kinase A signal transduction pathway and was effective in stratification of ligands by efficacy in both cell cultures (Glass and Northup, 1999) and tissue harvested from experimental animals (Griffin *et al*, 1998, 2001; Selley *et al*, 2001). However, there are a number of reagents and assay parameters which interact to determine the relative differences in efficacy, and variation across studies afforded relatively poor resolution to estimate relative efficacy when utilizing the body of literature. *In vivo*, both experimenter imposed constraints and the underlying neurobiology of the CB_1 receptor present challenges to determine efficacy for various endpoints.

1.6 CB_1 signal transduction

CB_1 receptors are seven transmembrane G-protein coupled receptors (GPCR) which classically signal through heteromeric G proteins consisting of α, β, and γ subunits. Upon ligand binding, a conformational change in the receptor occurs, spurring the dissociation of the α subunit from the βγ subunits as well as an exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP), likely mediated by a guanine exchange factor) at the α subunit. The dissociated α subunit then translocates to its target protein and exerts its action (Console-Bram *et al*, 2012). The signal is then terminated by the hydrolysis of bound GTP to GDP and the α and βγ subunits are once again complexed together to begin the cycle anew. More recently, GPCRs were found to simultaneously interact with multiple downstream signaling pathways in a ligand dependent manner, an emerging concept which has been termed "biased agonism" or "biased signaling" (Kenakin, 2015), although the concept has other names such as "functional

selectivity" or "stimulus trafficking". The downstream targets obviously depend on the expression and abundance of interacting proteins be they scaffolds or metabolically active thus these pathways are likely to be tissue or even cell specific (Kenakin, 2015). Signaling bias has been described for the CB_1 receptor for numerous ligands, although not all were described with a focus on elucidating the phenomenon. Therefore, current knowledge of signal transduction pathways should be considered with the likelihood that multiple pathways could be activated and the tissue and ligand inherently dictate the pattern of signaling which occurs.

CB_1 receptors are among the most abundant GPCRs in the human, non-human primate, and rodent CNS (Herkenham *et al*, 1990). They are typically coupled to $G\alpha_{i/o}$, thus they attenuate production of cyclic adenosine monophosphate (cAMP) via interactions with membrane bound adenylyl cyclase (Howlett, 1984; Melvin *et al*, 1993b). There are multiple subtypes of both $G\alpha_i$ (1,2, and 3) and $G\alpha_o$ (1 and 2) and differential signaling via $G\alpha_i$ or $G\alpha_o$ based upon the ligand occurs depending on the agonist used (Glass and Northup, 1999). WIN55,212-2 is distinct from other cannabinoids (THC, HU-210, CP55,940, 2-AG, methanandamide (mAEA), and cannabidiol) in its activation of the $G\alpha_q$-inositol phospate pathway to release intracellular calcium although WIN55,212-2 does so with relatively lower potency at $G\alpha_q$ compared to $G\alpha_{i/o}$ (Felder *et al*, 1995; Lauckner *et al*, 2005). When CB_1 is co-expressed with dopamine 2 (D2) receptors, its signaling can switch to $G\alpha_s$, thus increasing adenylyl cyclase activity to produce cAMP in a pertussis toxin insensitive manner (Glass and Felder, 1997; Jarrahian, 2003). This stimulatory effect may be due to heterodimerization with the D2 receptor (Kearn, 2005), as D2 receptor KO mice display diminished catalepsy following CP55,940 treatment (Andersson, 2005). The CB_1 receptor also interacts with G-protein activated inward rectifying potassium (GIRK) channels (Felder *et al*, 1995; McAllister *et al*, 1999) via

translocation of the βγ dimer following dissociation with the Gα subunit (Ho *et al*, 1999). WIN55,212 also may have effects on outward currents, though they seem to occur in a non-CB1 manner (Zhang *et al*, 2013).

Repeated administration of THC results G-protein receptor kinase (GRK) 2 and 4 expression and subsequent GRK-mediated phosphorylation of the intracellular surface of the CB_1 receptor (Rubino *et al*, 2006). This phosphorylation event then recruits beta arrestin 1 (BARR1) and beta arrestin 2 (BARR2) which can then result in internalization of receptors (Jin *et al*, 1999), or alternatively serve as a scaffold for proteins which phosphorylate extracellular signal-regulated kinase (ERK1/2) which seems to be inversely related to desensitization at the G-protein level (Daigle *et al*, 2008a). Genetic deletion of BARR2 increases sensitivity to the effects of THC relative to wild type mice, although CP55,940, methanandamide, and JWH-073, and O-1812 failed to produce the same differences (Breivogel *et al*, 2008). However, the lack of effects may be due to the high doses of each compound relative to reported ED_{50} values. Some experiments indicate downregulation drives the switch from Gα signaling to BARR2-ERK1/2 (Flores-Otero *et al*, 2014), while other studies posit that these signaling events occur simultaneous in a ligand-dependent manner (Laprairie *et al*, 2014). Interestingly, these two hypotheses are not mutually exclusive. Ligands could possess an initial bias for one pathway or another, and following chronic administration of the same agonist one pathway may become energetically unfavorable resulting in an alternative coupling to another pathway or pathways. In the context of cumulative dosing, a shift to the BARR2 signaling is entirely possible during testing though measuring this potential shift remains to be done.

1.7 Assay parameters and their effect on measuring efficacy in agonist stimulated GTPγS binding

The vast majority of studies conducted to differentiate cannabinoids by their efficacy employ the agonist-stimulated [^{35}S]GTPγS binding technique. Thus, these experiments served as a guide to select ligands to test in the whole animal. Though reliable, this *in vitro* technique may utilize a variety of reagents which may affect the sensitivity of the assay to detect the efficacy of ligands. In principle, agonist-stimulated [^{35}S]GTPγS binding measures the maximum possible stimulation of GDP for radiolabeled GTPγS in cells or membranes from biological samples, which triggers the dissociation of the Gα subunit from the βγ dimer. The Gα subunit signal is normally terminated by hydrolysis of GTP to GDP, yet the GTPγS resists this catabolism such that accumulation of bound GTPγS can be measured via a liquid scintillation counter. The relative concentrations of $MgCl_2$, GTPγS, GDP, Na^+, and membrane protein will each affect the resolution in measuring efficacy. Each of these reagents can be used within a wide range of concentrations and still produce the desired accumulation of GTPγS bound to G-proteins. The available literature reflects this considerable tolerance. E_{max} values for the widely used, high efficacy agonist CP55,940 varies from 136±11% above basal (Burkey *et al*, 1997a) to 200±7% (Hillard *et al*, 1999). In contrast, THC produces ~25% above basal (Burkey *et al*, 1997b) in some cases and in others produce no discernable stimulation (Griffin *et al*, 1998). Addition of adenosine deaminase (Moore *et al*, 2000) reduces basal activity by destroying ATP present in the tissue. This in turn increases resolution to distinguish high efficacy ligands by eliminating purinergic GPCR activity. A summary of cannabinoid agonist-stimulated GTPγS studies that includes reported E_{max} values of various ligands and assay conditions is included in Table 2.

Ligand	EC_{50} [nM]	SEM	Emax %net	SEM	$MgCl_2$ [mM]	Na^+ [mM]	GDP [μM]	GTPγS [nM]	GDP/ GTPγS	Tissue Conc. [μg/ml]	Species/Strain	Tissue Type	Reference
CP55,940	270	30	129.2	1.82	3	100	100	0.1	1000	300	C57BL6 mice	whole brain	Basavarajappa and Hungund, 1999
CP55,940	230	20	121.1	0.71	3	100	100	0.1	1000	300	DBA mice	whole brain	
HU-210	180	20	130	1.18	3	100	100	0.1	1000	300	C57BL6 mice	whole brain	
HU-210	160	10	120.6	2.5	3	100	100	0.1	1000	300	DBA mice	whole brain	
WIN55,212-2	330	10	155.4	4.08	3	100	100	0.1	1000	300	C57BL6 mice	whole brain	
WIN55,212-2	350	30	142.9	3.2	3	100	100	0.1	1000	300	DBA mice	whole brain	
WIN55,212-2	ND	ND	132	7	3	100	30	0.05	600	10 to 15	Sprague-Dawley	cerebellum	Breivogel et al., 1999
WIN55,212-2	ND	ND	115	18	3	100	30	0.05	600	10 to 15	Sprague-Dawley	hippocampus	
WIN55,212-2	ND	ND	249	48	3	100	30	0.05	600	10 to 15	Sprague-Dawley	striatum plus globus pallidus	
3 mM levonantrodol	ND	ND	620	36	3	100	30	0.05	600	10 to 15	Sprague-Dawley	cerebellum	Breivogel and Childers, 2000
3 mM levonantrodol	ND	ND	210	12	3	100	30	0.05	600	10 to 15	Sprague-Dawley	hippocampus	
3 mM levonantrodol	ND	ND	140	15	3	100	30	0.05	600	10 to 15	Sprague-Dawley	hypothalamus	
AEA	3600	2000	41	3	3	100	30	0.1	300	5	CB_1 KO	cerebellum	Breivogel et al., 2001
AEA	1400	300	150	7	3	100	30	0.1	300	5	CB_1 WT	cerebellum	
WIN55,212-2	1800	700	64	7	3	100	30	0.1	300	5	CB_1 KO	cerebellum	
WIN55,212-2	170	80	180	11	3	100	30	0.1	300	5	CB_1 WT	cerebellum	
CP55,940	ND	ND	149	13	3	100	30	0.1	300	10 to 30	Sprague-Dawley	cerebellum	Breivogel et al., 2003
HU-210	ND	ND	141	13	3	100	30	0.1	300	10 to 30	Sprague-Dawley	cerebellum	
mAEA	80	23	154	7	3	100	30	0.1	300	10 to 30	Sprague-Dawley	cerebellum	
THC	74	3	40	8	3	100	30	0.1	300	10 to 30	Sprague-Dawley	cerebellum	
WIN55,212-2	18	5	212	8	3	100	30	0.1	300	10 to 30	Sprague-Dawley	cerebellum	
AEA	846	84	96	5	2.5	150	50	0.1	500	not reported	Sprague-Dawley	whole brain	Burkey et al., 1997a
CP55,940	61.7	11.6	136	11	2.5	150	50	0.1	500	not reported	Sprague-Dawley	whole brain	
HU-210	2.26	0.38	123	4	2.5	150	50	0.1	500	not reported	Sprague-Dawley	whole brain	
THC	59		25		2.5	150	50	0.1	500	not reported	Sprague-Dawley	whole brain	

THC	70.9	31.7	37	2	2.5	150	50	0.1	500	not reported	Sprague-Dawley	whole brain	
WIN55,212-2	357		ND		2.5	150	50	0.1	500	not reported	Sprague-Dawley	whole brain	
2-AG	7585.78	1570.26	68	4	3	100	30	0.1	300	10	shrew	whole brain	Darmani et al., 2007
cannabidiol	ND	ND	ND	ND	3	100	30	0.1	300	10	shrew	whole brain	
CP55,940	5.25	1.33	105	1	3	100	30	0.1	300	10	shrew	whole brain	
HU-210	0.19	0.19	120	5	3	100	30	0.1	300	10	shrew	whole brain	
HU-211	ND	ND	ND	ND	3	100	30	0.1	300	10	shrew	whole brain	
mAEA	1230.27	650.81	120	25	3	100	30	0.1	300	10	shrew	whole brain	
SR141716A	ND	ND	ND	ND	3	100	30	0.1	300	10	shrew	whole brain	
THC	36.31	6.68	53	14	3	100	30	0.1	300	10	shrew	whole brain	
WIN55,212-2	107.15	98.58	104	8	3	100	30	0.1	300	10	shrew	whole brain	
5 mM WIN55,212-2	ND	ND	166.2	25.7	3	100	5	0.05	100	1 to 3	Wistar	cerebellum	Di Marzo et al., 2000
5 mM WIN55,212-2	ND	ND	163.3	11.6	3	100	5	0.05	100	1 to 3	Wistar	striatum	
5 mM WIN55,212-2	ND	ND	167	15.3	3	100	5	0.05	100	1 to 3	Wistar	hippocampus	
5 mM WIN55,212-2	ND	ND	147.9	25.9	3	100	5	0.05	100	1 to 3	Wistar	cerebral cortex	
5 mM WIN55,212-2	ND	ND	112.4	12.5	3	100	5	0.05	100	1 to 3	Wistar	limbi forebrain	
5 mM WIN55,212-2	ND	ND	94.8	14.7	3	100	5	0.05	100	1 to 3	Wistar	brainstem	
AEA	538	54	71	6	1	100	4	0.4-0.8	50	20 to 40	NA	Sf9 cells-Gi	Glass and Northup, 1999
AEA	776	78	45	2	1	100	4	0.4-0.8	50	20 to 40	NA	Sf9 cells-Go	
HU-210	2.3	0.3	100	ND	1	100	4	0.4-0.8	50	20 to 40	NA	Sf9 cells-Gi	
HU-210	3.1	0.3	100	ND	1	100	4	0.4-0.8	50	20 to 40	NA	Sf9 cells-Go	
THC	196	13	64	10	1	100	4	0.4-0.8	50	20 to 40	NA	Sf9 cells-Gi	
THC	185	28	42	0.4	1	100	4	0.4-0.8	50	20 to 40	NA	Sf9 cells-Go	
WIN55,212-2	330	53	72	12	1	100	4	0.4-0.8	50	20 to 40	NA	Sf9 cells-Gi	
WIN55,212-2	362	93	65	3	1	100	4	0.4-0.8	50	20 to 40	NA	Sf9 cells-Go	
AEA	ND	ND	ND	ND	3	150	100	0.05	2000	2	Sprague-Dawley	cerebellum	Griffin et al., 1998
cannabinol	ND	ND	ND	ND	3	150	100	0.05	2000	2	Sprague-Dawley	cerebellum	
CP55,244	0.47	0.25	165	17	3	150	100	0.05	2000	2	Sprague-Dawley	cerebellum	

CP55,940	ND	ND	61	28	3	150	10	0.05	200	2	Sprague-Dawley	cerebellum	
CP55,940	17.57	10.54	114	17	3	150	100	0.05	2000	2	Sprague-Dawley	cerebellum	
deoxy-HU-210	9.6	8.5	150	55	3	150	100	0.05	2000	2	Sprague-Dawley	cerebellum	
fluoro-mAEA	25.37	20.39	97	42	3	150	100	0.05	2000	2	Sprague-Dawley	cerebellum	
HU-210	0.55	0.3	140	23	3	150	100	0.05	2000	2	Sprague-Dawley	cerebellum	
JWH-030	82.4	68.4	56	19	3	150	100	0.05	2000	2	Sprague-Dawley	cerebellum	
JWH-073	104.8	87.86	29	20	3	150	100	0.05	2000	2	Sprague-Dawley	cerebellum	
O-1064	246.2	233.75	60	55	3	150	100	0.05	2000	2	Sprague-Dawley	cerebellum	
THC	ND	ND	51	7	3	150	10	0.05	200	2	Sprague-Dawley	cerebellum	
THC	ND	ND	ND	ND	3	150	100	0.05	2000	2	Sprague-Dawley	cerebellum	
WIN55,212-2	ND	ND	89	14	3	150	10	0.05	200	2	Sprague-Dawley	cerebellum	
WIN55,212-2	151.1	50.5	156	12	3	150	100	0.05	2000	2	Sprague-Dawley	cerebellum	
Δ8-THC	0	ND	0	ND	9	150	100	0.05	2000	2	Sprague-Dawley	cerebellum	Griffin et al., 2001
O-1125	165	12.8	17.4	6	9	150	100	0.05	2000	2	Sprague-Dawley	cerebellum	
O-1176	0	ND	0	ND	9	150	100	0.05	2000	2	Sprague-Dawley	cerebellum	
O-1184	0	ND	0	ND	9	150	100	0.05	2000	2	Sprague-Dawley	cerebellum	
O-1236	87.5	9.7	16.6	5	9	150	10	0.05	200	2	Sprague-Dawley	cerebellum	
O-1237	51.3	5.5	10.6	8	9	150	10	0.05	200	2	Sprague-Dawley	cerebellum	
O-1238	58.3	8.5	29.7	15	9	150	100	0.05	2000	2	Sprague-Dawley	cerebellum	
O-584	0	ND	0	ND	9	150	100	0.05	2000	2	Sprague-Dawley	cerebellum	
O-806	0	ND	0	ND	9	150	100	0.05	2000	2	Sprague-Dawley	cerebellum	
O-823	0	ND	0	ND	9	150	100	0.05	2000	2	Sprague-Dawley	cerebellum	
11-OH-Δ9-THC	94	6	132	4	5	150	10	0.65	15.38	not reported	Sprague-Dawley	cerebellum	Kearn et al., 1999
AEA	276	53	166	6	5	150	10	0.65	15.38	not reported	Sprague-Dawley	cerebellum	
CP55,940	29.6	11	199	7	5	150	10	0.65	15.38	not reported	Sprague-Dawley	cerebellum	
HU-210	4.3	1.7	179	13	5	150	10	0.65	15.38	not reported	Sprague-Dawley	cerebellum	
THC	91	32	126	17	5	150	10	0.65	15.38	not reported	Sprague-Dawley	cerebellum	
WIN55,212-2	102	28	217	8	5	150	10	0.65	15.38	not reported	Sprague-Dawley	cerebellum	

AEA	490	ND	134	3	3	0	30	0.5	60	10 to 15	CB_1 KO	cerebellum	Monory et al., 2002
AEA	3040	ND	343	29	3	0	30	0.5	60	10 to 15	CB_1 WT	cerebellum	
HU-210	ND	ND	ND	ND	3	0	30	0.5	60	10 to 15	CB_1 KO	cerebellum	
HU-210	17	ND	492	24	3	0	30	0.5	60	10 to 15	CB_1 WT	cerebellum	
THC	ND	ND	ND	ND	3	0	30	0.5	60	10 to 15	CB_1 KO	cerebellum	
THC	1490	ND	171	13	3	0	30	0.5	60	10 to 15	CB_1 WT	cerebellum	
WIN55,212-2	1780	ND	206	16	3	0	30	0.5	60	10 to 15	CB_1 KO	cerebellum	
WIN55,212-2	1720	ND	444	21	3	0	30	0.5	60	10 to 15	CB_1 WT	cerebellum	
11-hydroxy-THC	110	50	162	6	1	0	50	0.2	250	30	rat	cerebellum	Petitet et al., 1997
11-nor-Δ8-THC-9-carboxylic acid	>10000	ND	ND	ND	1	0	50	0.2	250	30	rat	cerebellum	
11-nor-Δ9-THC-9-carboxylic acid	>10000	ND	ND	ND	1	0	50	0.2	250	30	rat	cerebellum	
AEA	2300	1100	186	12	1	0	50	0.2	250	30	rat	cerebellum	
cannabinol	170	30	130	2	1	0	50	0.2	250	30	rat	cerebellum	
CP55,940	9	3	200	2	1	0	50	0.2	250	30	rat	cerebellum	
mAEA	180	80	204	14	1	0	50	0.2	250	30	rat	cerebellum	
THC	530	310	154	8	1	0	50	0.2	250	30	rat	cerebellum	
WIN55,212-2	99	45	175	10	1	0	50	0.2	250	30	rat	cerebellum	
WIN55,212-3	>10000	ND	ND	ND	1	0	50	0.2	250	30	rat	cerebellum	
cannabidiol	0	ND	0	ND	1	0	100	0.2	500	30	Sprague-Dawley	cerebellum	Petitet et al., 1998
cannabinol	187	ND	24	ND	1	0	100	0.2	500	30	Sprague-Dawley	cerebellum	
CP55,940	53	ND	84	ND	1	0	100	0.2	500	30	Sprague-Dawley	cerebellum	
THC	216	ND	54	ND	1	0	100	0.2	500	30	Sprague-Dawley	cerebellum	
WIN55,212-2	ND	ND	140.25	10.15	3	100	50	0.1	500	?	Sprague-Dawley	medial prefrontal cortex	Rodríguez-Gaztelumendi et al., 2009
WIN55,212-2	ND	ND	173.71	12.63	3	100	50	0.1	500	?	Sprague-Dawley	caudate-putamen	
WIN55,212-2	ND	ND	171.04	26.71	3	100	50	0.1	500	?	Sprague-Dawley	hippocampus	
WIN55,212-2	ND	ND	110.07	2.98	3	100	50	0.1	500	?	Sprague-Dawley	amygdala	
WIN55,212-2	ND	ND	105.55	5.26	3	100	50	0.1	500	?	Sprague-Dawley	dorsal raphe nucleus	

mAEA	114	4.2	542	84	3	100	30	0.08	375	5 to 10	CB_1 HET	cerebellum	Selley et al., 2001
mAEA	63	0.4	277	36	3	100	30	0.08	375	5 to 10	CB_1 HET	hippocampus	
mAEA	33	4.9	551	103	3	100	30	0.08	375	5 to 10	CB_1 HET	striatum plus globus pallidus	
mAEA	ND	ND	ND	ND	3	100	30	0.08	375	5 to 10	CB_1 HET	cingulate cortex	
mAEA	160	14	375	23	3	100	30	0.08	375	5 to 10	CB_1 WT	cerebellum	
mAEA	96	6.9	265	33	3	100	30	0.08	375	5 to 10	CB_1 WT	hippocampus	
mAEA	67	5.6	227	47	3	100	30	0.08	375	5 to 10	CB_1 WT	striatum plus globus pallidus	
mAEA	56	3.6	183	42	3	100	30	0.08	375	5 to 10	CB_1 WT	cincgulate cortex	
THC	34	7.7	215	69	3	100	30	0.08	375	5 to 10	CB_1 HET	cerebellum	
THC	24	3.2	63	24	3	100	30	0.08	375	5 to 10	CB_1 HET	hippocampus	
THC	12	2.1	220	94	3	100	30	0.08	375	5 to 10	CB_1 HET	striatum plus globus pallidus	
THC	ND	ND	ND	ND	3	100	30	0.08	375	5 to 10	CB_1 HET	cingulate cortex	
THC	51	5.7	153	41	3	100	30	0.08	375	5 to 10	CB_1 WT	cerebellum	
THC	44	1.7	55	31	3	100	30	0.08	375	5 to 10	CB_1 WT	hippocampus	
THC	23	2.7	92	44	3	100	30	0.08	375	5 to 10	CB_1 WT	striatum plus globus pallidus	
THC	28	4	93	17	3	100	30	0.08	375	5 to 10	CB_1 WT	cingulate cortex	
WIN55,212-2	204	18	167	26	3	100	30	0.08	375	5 to 10	CB_1 HET	cerebellum	
WIN55,212-2	134	5.5	176	12	3	100	30	0.08	375	5 to 10	CB_1 HET	hippocampus	
WIN55,212-2	60	5.7	204	27	3	100	30	0.08	375	5 to 10	CB_1 HET	striatum plus globus pallidus	
WIN55,212-2	106	7.9	287	43	3	100	30	0.08	375	5 to 10	CB_1 HET	cingulate cortex	
WIN55,212-2	248	29	104	18	3	100	30	0.08	375	5 to 10	CB_1 WT	cerebellum	
WIN55,212-2	181	14	137	19	3	100	30	0.08	375	5 to 10	CB_1 WT	hippocampus	
WIN55,212-2	112	13	126	17	3	100	30	0.08	375	5 to 10	CB_1 WT	striatum plus globus pallidus	
WIN55,212-2	139	3.3	178	220	3	100	30	0.08	375	5 to 10	CB_1 WT	cingulate cortex	
WIN55,212-2	0.04	0.01	119	1.5	3	100	30	0.1	300	3.5 to 8	Long-Evans female adolescent	prefrontal cortex	Wiley et al., 2008
WIN55,212-2	0.06	0.03	102	1.8	3	100	30	0.1	300	3.5 to 8	Long-Evans female adolescent	striatum	

WIN55,212-2	0.19	0.02	87	10.6	3	100	30	0.1	300	3.5 to 8	Long-Evans female adolescent	midbrain
WIN55,212-2	0.08	0.02	205	10	3	100	30	0.1	300	3.5 to 6	Long-Evans female adult	prefrontal cortex
WIN55,212-2	0.13	0.02	194	5.1	3	100	30	0.1	300	3.5 to 6	Long-Evans female adult	striatum
WIN55,212-2	0.08	0.03	137	8.5	3	100	30	0.1	300	3.5 to 6	Long-Evans female adult	midbrain
WIN55,212-2	0.16	0.01	112	5.4	3	100	30	0.1	300	3.5 to 9	Long-Evans male adolescent	prefrontal cortex
WIN55,212-2	0.14	0.02	96	5.1	3	100	30	0.1	300	3.5 to 9	Long-Evans male adolescent	striatum
WIN55,212-2	0.14	0.01	105	5.8	3	100	30	0.1	300	3.5 to 9	Long-Evans male adolescent	midbrain
WIN55,212-2	0.88	0.38	135	2.9	3	100	30	0.1	300	3.5 to 7	Long-Evans male adult	prefrontal cortex
WIN55,212-2	1.46	0.45	100	2.6	3	100	30	0.1	300	3.5 to 7	Long-Evans male adult	striatum
WIN55,212-2	0.41	0.17	91	5.1	3	100	30	0.1	300	3.5 to 7	Long-Evans male adult	midbrain

Table 1. Assay conditions of agonist stimulated [^{35}S]GTPγS binding experiments as determinants of E_{max} estimations. Concentrations of MgCl2, Na+, relative concentrations of GDP and GTPγS, strain, and selected CNS region all influence the magnitude of measured efficacy. Relative differences in efficacy, especially at either extreme, are highly dependent on the interplay of each reagent.

Intracellular Na^+ allosterically inhibits G-protein mediated signaling stimulated by dopaminergic, opioidergic, and serotonergic, and many other GPCRs (Katritch *et al,* 2014). CB_1 receptors are similarly regulated by Na^+ via the same evolutionarily conserved aspartate residue on the second transmembrane helix (Tao and Abood, 1998). While mutation of the sodium-binding aspartate appears to affect binding affinity of certain ligands, such as desacetyllevonantrodol (Houston and Howlett, 1998), it nonetheless reduces downstream inhibition of adenylyl cyclase resulting in greater than expected cAMP formation for WIN55,212-2, CP55,940, AEA, and THC (Tao and Abood, 1998). These results suggest the existence of an inverse relationship between the concentration of Na^+ and the E_{max} measured by agonist-stimulated [^{35}S]GTPγS binding. Some experiments report no Na^+ in the assay (Petitet *et al*, 1998), though most are within the 100-150 mM range (Breivogel and Childers, 2000; Griffin *et al*, 1998), a physiologically relevant concentration.

Magnesium ions (Mg^{2+}) are important co-factors for the binding of both GDP and GTP to the G-protein and typical concentrations in agonist-stimulated [^{35}S]GTPγS binding typically range from 1 mM (Petitet *et al*, 1997) to 5 mM (Thomas *et al*, 2005), though one study reports using 9 mM in the GTPγS assay buffer (Griffin *et al*, 1999). In the absence of Mg^{2+} the affinities of GDP and GTP are drastically reduced, and if the concentrations of both are sufficiently low, G-proteins become unstable and may degrade (Sprang, 1997). Magnesium also extends the duration of GTPγS binding (Higashijima *et al*, 1987); thus manipulating this ion can enhance the sensitivity to measure accumulation via scintillation counting.

The quantity of protein for agonist-stimulated [^{35}S]GTPγS binding may also affect the measured E_{max} value. A high protein concentration should increase the relative concentration of CB_1. Accordingly, the Stephenson equation predicts a positive relationship between protein

concentration and the maximum effect. Thus, ligands appear indistinguishable regardless of efficacy in preparations containing a large receptor reserve. As the density of CB_1 expression varies with CNS region, different brain regions are often used to change the relative concentration of the receptor. All other factors being equivalent, the choice of protein concentration need only be sufficiently high to detect stimulation and not so high that it precludes differentiation. Thus, comparisons made between studies utilizing the same drugs, but different tissues, will likely yield calculated different E_{max} values. However, a series of ligands varying in efficacy assessed in different tissues may be comparable in a qualitative manner. Typical protein concentrations range from as low as 2 μg/ml (Griffin *et al,* 1998) to as high as 30 μg/ml (Petitet *et al*, 1998). The brain region most utilized in *in vitro* cannabinoid studies is the cerebellum (Table 2), likely due to the ease of dissection and its relatively high expression of CB_1. It also possess rather low endogenous basal activity, which bolsters the resolution to detect small, but significant, increases in binding of low efficacy cannabinoids. If the anatomical distribution of the functional activity of CB_1 in the presence of an agonist is desired, agonist-stimulated [^{35}S]GTPγS autoradiography is of great value (Sim *et al*, 1996a). However, this technique does not readily lend itself to testing a large range of drugs under the same conditions, as is the case with agonist-stimulated [^{35}S]GTPγS binding. Autoradiography does provide exquisite neuroanatomical detail but only one test ligand may be utilized per sample. For agonist-stimulated [^{35}S]GTPγS binding, samples are often distributed over many assay wells or tubes, thus affording the flexibility to assess the effect of many dose-effect curves in the same tissue. Importantly, this design allows quantitative inferences to be made regarding the pharmacological properties of the drugs tested, specifically as they relate to GDP-GTP exchange.

The relative concentration of GDP to [^{35}S]GTPγS can be easily manipulated and represents a highly useful assay parameter to vary in order to increase the resolution of the binding assay. Progressively increasing concentrations of GDP drives down basal activity (Sim *et al*, 1995), but facilitates detecting differences in efficacy for ligands clustered near the top of the continuum. Conversely, very low concentrations of GDP favor differentiation between agonists of very low efficacy, such as THC and analogs (see Table 2, Griffin et al., 2001). As with the tissue concentration, the range of GDP employed across studies is quite wide, ranging from 4 μM (Glass and Northup, 1999) to 100 μM (Griffin *et al*, 1998). As predicted, low concentrations do not as readily distinguish between high efficacy ligands, while high concentrations more easily separate high efficacy ligands (Griffin *et al*, 1998). The [^{35}S]GTPγS concentration in these assays often falls in the sub-nanomolar range, rendering the ratio of GDP/[^{35}S]GTPγS anywhere from 15 (Kearn *et al*, 1999) to 2000 (Griffin *et al*, 2001). Both GDP and GTP have nanomolar affinities for most G-proteins, with GTP having approximately 1-10 fold higher affinity depending on the G-protein type and/or subtype (Sprang, 1997). [^{35}S]GTPγS has an even greater affinity for the G-protein that may, in part, account for the large discrepancy in relative concentrations, which are necessary to detect differences among ligands. Additionally, binding of a ligand to the CB_1 receptor reduces the apparent affinity of GDP for G-proteins and increases that of GTPγS (Breivogel *et al*, 1998).

To effectively assess whether CB_1 ligands differ in efficacy, the assay parameters must be able to capture agonist stimulation of CB_1 activity for both high and low efficacy compounds. The NaCl (100 mM) and $MgCl_2$ (3mM) should be sufficiently high to reduce basal activity and extend the time course of GTPγS binding, respectively. Incubation with adenosine deaminase will metabolize much of the present ATP, reducing purinergic GPCR activity and reducing the

non-CB_1 activity further. Concentrations of GTPγS (0.1 nM) and GDP (30 μM) will reflect a moderate ratio of GDP to GTPγS (300) to facilitate sensitivity to both high and low efficacy compounds. This moderate concentration would likely reduce sensitivity to ligands with extremely high or low efficacy. However, this experimental window can be altered by varying the GDP concentration, and concomitantly altering the ratio of GDP to GTPγS. In this thesis, I captured respective high and low receptor conditions by using cerebellum and spinal cord samples. Additionally, receptor levels were further varied through the employment of CB_1 WT, HET, and KO mice will be used. The overall protein concentrations were held constant irrespective of genotype and brain region to ensure observed differences are the result of relative changes in CB_1 expression, as well as to enable quantitative comparisons between brain regions and genotypes.

1.8 Selection of cannabinoid ligands and controls based upon evidence from previous [^{35}S]GTPγS binding experiments

The ligands THC, CP47,497, JWH-073, CP55,940, and WIN55,212-2 were selected for study in the cumulative dosing triad and agonist-stimulated [^{35}S]GTPγS binding due to their apparent relative differences in efficacy, as reviewed in Table 2. CP47,497 and JWH-073 were also selected because of their historical detections in abused preparations of Spice and K2 products (Auwärter *et al*, 2009). To aid in these studies, the heat degradant of the highly efficacious A-834,375 (A-834,735D) was selected as another high efficacy cannabinoid ligand. Preliminary data indicate the degradant form has very high efficacy at the CB_1 receptor in recombinant human CB_1 (hCB_1) expressing human embryonic kidney (HEK) cells, and it will serve as an archetypal representative of newer SC compounds (Thomas and Wiley, 2014). Preliminary data from our own lab also indicated CP47,497 was lower in efficacy than

CP55,940. Although many of these ligands have been tested under the same conditions, the assays were often not optimized to detect differences between both and low efficacy ligands, and only WIN55,212-2 and THC were tested in CB_1 KO tissue. Unlike most other cannabinoid agonists, WIN55,212-2 stimulates [^{35}S]GTPγS binding in these tissues suggesting that this non-CB_1 activity yields an over-estimation of reported E_{max} values. Indeed, reported studies do not account for the non-CB_1 stimulation (Monory *et al*, 2002). The potential off-target stimulation is especially important to consider in the case of the only agonist-stimulated [^{35}S]GTPγS binding study conducted utilizing CB_1 HET mice to manipulate receptor density (Selley *et al*, 2001). The same study also utilized methanandamide, which may stimulate [^{35}S]GTPγS binding in a similar fashion as the structurally related endocannabinoid anandamide (Breivogel *et al*, 2001; Monory *et al*, 2002). Accordingly, the estimated E_{max} values reported in WT and HET membranes might be equally inflated, rendering the relative efficacies closer to one another than when non-CB_1 stimulation is subtracted. In the studies reported in this thesis (Chapter 2), the same concentrations of agonists will be used in both WT and HET samples, while KO tissue will be used to detect potential non-CB_1 stimulation. Each of the ligands selected has similar affinities for the CB_1 receptor and the CB_2 receptor. However, it is important to note that CB_2 receptor mRNA is expressed at very low levels within the CNS, and likely contributes little to the overall signal. In contrast, CB_1 mRNA and protein are vastly abundant (Galiègue *et al*, 1995) and represents among the highest expressed GPCRs in the brain. Therefore, CB_2 contribution to the signal is negligible and off target-stimulation is likely to reflect a heretofore unknown target or targets.

For *in vivo* experiments, the selectivity of the assay for cannabimimetic agents was assessed using the mu opioid receptor agonist morphine and the atypical antipsychotic

chlorpromazine. Despite possessing non-CB_1 mechanisms of action, these drugs produce a subset of the triad endpoints. Importantly, both morphine and chlorpromazine were expected to be equipotent to produce their effects irrespective of genotype, indicating the specificity for CB_1 activity.

1.9 Potential methods to determine efficacy at the CB_1 receptor *in vivo*

CB_1 receptors are heterogeneously expressed throughout the mammalian brain, with high receptor concentrations in brain regions believed to mediate the pharmacological effects of cannabis and THC (Herkenham *et al*, 1990, 1991). Many cannabimimetic effects occur via this receptor (Ledent *et al*, 1999; Rinaldi-Carmona, 1994; Rinaldi-Carmona *et al*, 1995; Zimmer *et al*, 1999a), including catalepsy, hypothermia, antinociception, and hypolocomotion. Consequentially, the receptor reserve mediating the pharmacological actions of cannabinoids may be sufficient for even very low efficacy compounds produce effects a similar magnitude to high efficacy agonists across many endpoints. This inability to distinguish efficacy due to dense CB_1 receptor expression presents unique challenges to ascertain *in vivo* efficacy at the CB_1 receptor, especially with the knowledge that CB_1 receptor expression varies considerably across brain regions (Herkenham *et al*, 1990). The requisite reduction in CB_1 receptors available for signaling may be achieved through pharmacological and/or genetic approaches. In the following discussion, three general experimental approaches are discussed in which CB_1 expression is experimentally reduced to investigate efficacy.

First, repeated administration of THC and synthetic cannabinoids leads to tolerance and cross tolerance to other cannabinoids (Fan *et al*, 1994; Pertwee *et al*, 1993). This tolerance occurs concomitantly with receptor downregulation and desensitization which occurs in a brain region specific manner, as determined by radioligand and agonist-stimulated [^{35}S]GTPγS

autoradiography (Lazenka *et al*, 2014; Tai *et al*, 2015a). Potency shifts may be used to infer differences in efficacy following chronic treatment in the case of drug discrimination (Hruba *et al*, 2012), though the neural substrates, which underlie this behavior, are not well understood. Other studies which demonstrate varying degrees of tolerance depending on which agonist is repeatedly administered also may not readily differentiate between efficacy of the test drugs, and the duration and timing of the dosing of the toleragen are determining factors for the degree of tolerance (Fan *et al*, 1994). Another limitation of this approach may lie within the inherent differences in ligand bias, which almost assuredly exist among cannabinoid agonists (Laprairie *et al*, 2014). BARR2 plays a role in both acute CB_1-mediated effects of THC (Breivogel *et al*, 2013) and tolerance following repeated administration (Nguyen *et al*, 2012). BARR2 also promotes CB_1-mediated signaling via ERK1/ERK2 (Franklin *et al*, 2013); thus, observed tolerance may be the result of decreased $G\alpha_{i/o}$ signaling and a shunting towards BARR2. In cell cultures, ERK1/ERK2 signaling elevates during prolonged CP55,940 exposure suggesting this may be the case (Daigle *et al*, 2008a). Unsurprisingly, this apparent shunting of pathways likely occurs in a CNS region dependent manner (Rubino *et al*, 2006), further complicating interpretations of chronic dosing regimens as they relate to efficacy. Chronic THC treatment induced changes in BARR2 and ERK/Ras signaling in striatum and cerebellum, but not in hippocampus or prefrontal cortex, and the desensitization observed may occur via alternate mechanisms (Rubino *et al*, 2006).

A second pharmacological approach to infer efficacy involves the use of an irreversible antagonist to progressively and dose-dependently reduce the number of receptors available. Irreversible antagonists are associated with insurmountable rightward and downward shifts in dose response curves. The reduction in receptor population in turn facilitates the estimation of τ,

or $\frac{R_T}{K_E}$, via isolation of K_E, or the concentration of the agonist receptor complex to produce a 50% effect, as seen in the operational model (Black and Leff, 1983; Kenakin, 2014). As R_T approaches zero, the EC_{50} will collapse upon the K_A. This approach has been demonstrated utilizing the irreversible mu opioid antagonist clocinnamox to estimate the τ of various mu opioid ligands (Pawar *et al*, 2007). However, this approach is not feasible to investigate efficacy of compounds at CB_1, as no irreversible antagonist is available yet for this receptor. On the other hand, the anandamide analog AM3577, which binds covalently to the orthosteric site and inhibits adenylyl cyclase (Janero *et al*, 2015), may provide a tool for this sort of approach. However, AM3577 would unlikely be able to distinguish between agonists of lower or similar efficacy.

Genetic approaches represent third way to reduce CB_1 expression. Short interfering ribonucleic acid (siRNA) represents another potential method to reduce the overall expression of CB_1, though the degree of knockdown may be challenging to be implemented in systematic and controlled manner throughout the entire CNS. Alternatively, the use of CB_1 wild type (WT), heterozygous (HET), and knockout (KO) transgenic mice reflects a simple tool to reduce receptor levels in a controlled fashion (Ledent *et al*, 1999; Zimmer *et al*, 1999b). WT mice express the normal abundance of CB_1, HET mice express approximately half of normal levels (Selley *et al*, 2001), and KO do not express functional CB_1 (Zimmer *et al*, 1999b). Thus, these transgenic mice represent a way to assess the loss of potency and/or efficacy of different compounds at CB_1, while simultaneously maintaining sensitivity to potential off target effects. This approach has several advantages versus the alternative methods described above. First, the reductions in receptor population are presumably constitutive and stable. Second, the reduction in CB_1 expression is proportional to the tissue. While CB_1 receptor density varies considerably with brain region (Herkenham *et al*, 1990), the expression in HET mice is approximately half of

WT levels in cerebellum, hippocampus, striatum, and cingulate cortex (Selley *et al*, 2001), and this pattern of half expression in HET mice presumably continues throughout the brain. A limitation of this approach is that it does not provide the graded reduction in receptor an irreversible antagonist would afford. Accordingly, the estimation of K_E is not possible with a great degree of confidence. Instead, a simple formulation of the receptor theory equation may predict the pharmacological effects of agonists varying in CB_1 efficacy.

1.10 Theoretical predictions based upon the Stephenson receptor theory model

$$E = [R_T] * [\varepsilon] * \frac{[A]}{[A] + K_D}$$

E = maximum effect

R_T = receptor population

ε = efficacy of the drug

$\frac{[A]}{[A]+ K_D}$ = fractional occupancy

The Stephenson equation (Stephenson, 1956) is an extension of the Furchgott receptor occupancy equation, but it includes the term "ε" which acknowledges the inherent differences in ligands in terms of the degree of activation they are able to produce. This equation also determines the maximum amount of activation an individual ligand can stimulate which accounts for the existence of partial agonists. The fractional occupancy term $\frac{[A]}{[A]+K_D}$ produces the characteristic hyperbolic (in a linear-linear scale) and sigmoidal (in a log-linear scale) shapes seen in dose-effect studies. Importantly, when [A] = K_D then the fractional occupancy is half which in turn reflects the equilibrium between bound and unbound ligand. The affinity and efficacy terms determine potency insofar as drugs which are more efficacious or possess higher

affinity also tend to be more potent, with the most potent drugs tending to have high efficacy and high affinity. The other determinant of the effect of a drug is the number of receptor available to signal through. The number of receptors may vary from tissue from tissue, and in the case of cannabinoids definitely does. Another implication of this variance in receptor expression is differential tissue sensitivity given the same ligand. In the case of CB_1 transgenic mice, the consequences of 100%, 50%, and 0% receptor expression may be modeled and explored (Figure 6).

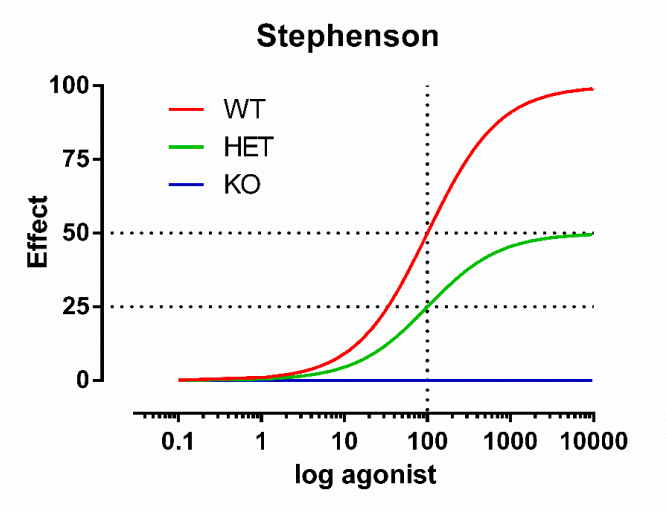

Figure 6. Utilizing the Stephenson receptor theory equation, predictions regarding changes in efficacy and potency regarding reductions in receptor populations were depicted. In WT tissue, which expresses normal levels of CB_1, maximal activation was achieved. In HET tissue, exactly half of WT activation occurred and in KO tissue no activation was observed. This activation corresponds with 100%, 50%, and 0% expression of CB_1. At a given dose of the agonist, the fractional occupancy in WT and HET tissues were identical.

Using a single agonist in each genotype, the maximum effect is assumed to be proportional to the reduction in total receptor number. Accordingly, a given CB_1 agonist is predicted to possess the following efficacies in the three genotypes, assuming no receptor reserve: 1) WT mice that possess 100% of the receptors will show a maximum effect; 2) HET mice, which possess 50% of the receptors, a concordant 50% reduction in efficacy will be observed; 3) KO mice, which have no receptors available for signaling, will not show any measurable effect regardless concentration. However, the proportion of the available receptors occupied does not change for a given concentration, as indicated by the vertical dotted line in Figure 6. At the EC_{50} in WT mice half of 100% of the receptors are occupied whereas half of 50% of the receptors are occupied in HET mice. There is no occupancy in the KO mice as there are no receptors. It should be noted that these conditions do not allow for reliable estimation of K_E as derived from the Operation Model as only two EC_{50} concentrations are observed, obviating the necessary nonlinear regression necessary to calculate K_E.

The pharmacological effects of cannabinoids are mediated by distinct pools of CB_1 receptors. Moreover, the concentration of CB_1 receptors are known to vary throughout the CNS. Intuitively, pharmacological effects mediated by circuits containing very high CB_1 expression were hypothesized to show very small shifts between WT and HET mice (Figure 7). Under conditions of moderate receptor levels, more resolution is gained to distinguish between low and high efficacy ligands in which rightward and downward shifts may be readily observed with low efficacy ligands (Figure 8). Very low receptor expression conditions may further differentiate between ligands of varying efficacy when compared amongst the genotypes, but the resolution to do so may be lost as the dose-effect relationships begin to congregate towards the abscissa (Figure 9). For instance, ligands that both possess low, but different levels of efficacy could be

tested under both moderate and low receptor conditions. The moderate expression may differentiate between the efficacies of the two test ligands whereas the low receptor conditions would display a floor effect, preempting differentiation under those conditions. Consequently, it remains important to assess efficacy under a variety of conditions to properly ascertain actual differences in efficacy.

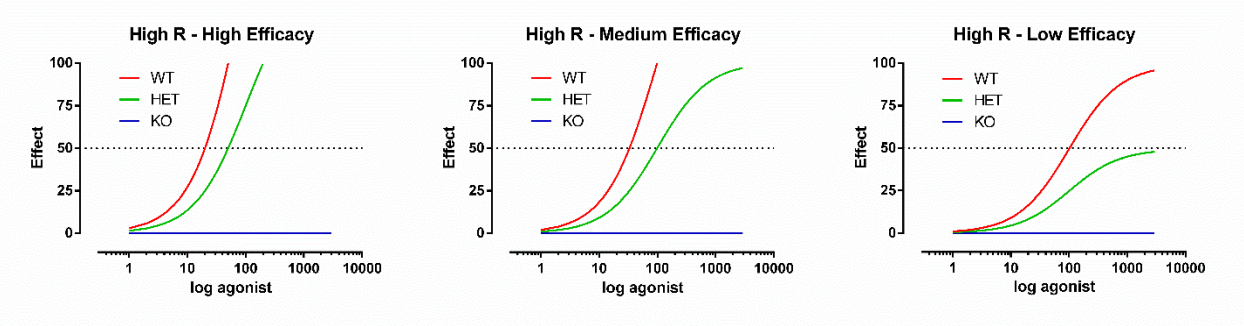

Figure 7. High receptor expression conditions revealed only small changes in potency between WT and HET dose-effect curves utilizing ligands with high or medium efficacy. Larger shifts were observed with low efficacy ligands, but maximal effects were achieved in WT but not HET mice.

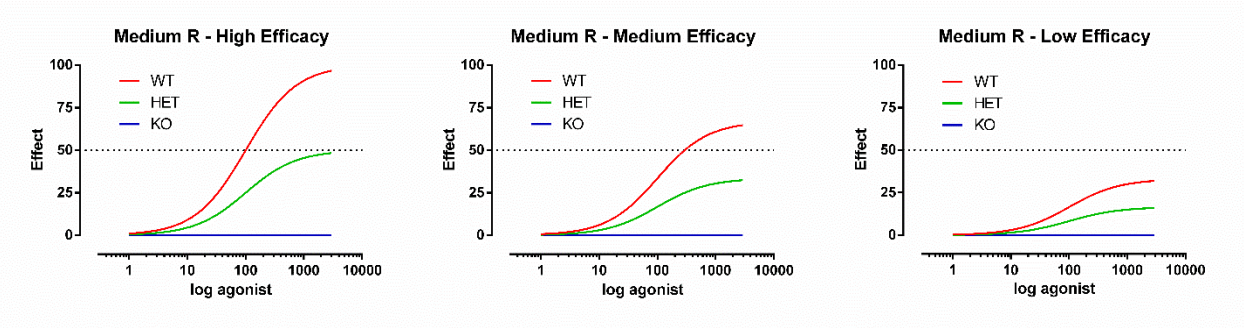

Figure 8. Moderate levels of CB1 expression show better resolution to distinguish between ligands that vary in efficacy utilizing differential potency between WT dose effect curves. Additionally, downward shifts are readily observed across ligands which vary in efficacy due to a 50% reduction in receptor population.

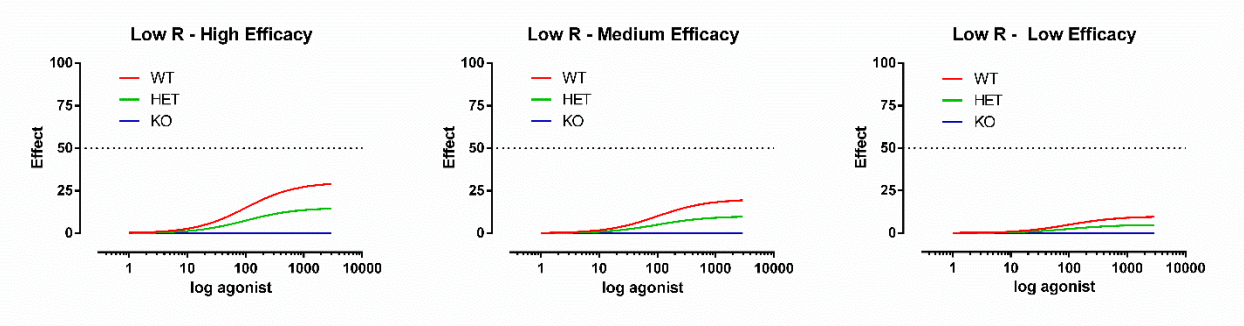

Figure 9. Low receptor conditions differentiate high efficacy ligands more readily than low efficacy ligands. When low efficacy ligands are utilized under low receptor expression conditions, dose-effect curves collapse on the abscissa. In practice, experimental error may eclipse a potential differences between genotypes.

Practical and experimentally imposed limits of detection also influence the ability to stratify ligands based upon efficacy. The experimental floor may be a function of the sensitivity of the assay and/or tissue, or an experimenter imposed minimum value. In situations in which the floor is very high, the EC_{50} estimate will be shifted to the right relative to the theoretical (Figure 10). The experimental ceiling is usually arbitrarily determined and may not be based upon the true maximum effect that might be achieved by various agonists. For example, in assays that can evoke tissue damage (e.g. warm water tail withdrawal and radiant heat tail-flick tests), a cutoff time is used for animal welfare concerns. Practical considerations such as the length of the test can also contribute, as in some cases additional observation does not yield a substantial degree of additional information. In cases where the ceiling is very low relative to the maximum observable effect, the potency of agonists which do achieve a maximum effect will shift to the left relative to the true measurable maximum (Figure 11). Although ceiling and floor effects represent challenges in deriving potency estimations, as long as the same assay conditions are applied for each agonist then the relative rank order efficacy among the agonists does not change. Provided the observable effects capture a sufficient portion of each dose-effect relationship, then whatever potency estimations are calculated will reflect the interrelationships between the agonists. In the context of using CB_1 transgenic mice that express different concentrations of receptors, ceiling effects likely impose the largest restrictions on measuring actual differences in maximum effect. Although the maximum effect in HET mice will be half of what is observed in WT mice, the experimental window may afford maximum effects in both genotypes with the only apparent difference being potency. Therefore, the potency shift between the WT and HET mice may reflect efficacy differences, though E_{max} values may not differ. The shift in potency, however, would not reflect non-CB_1 mediated contributions.

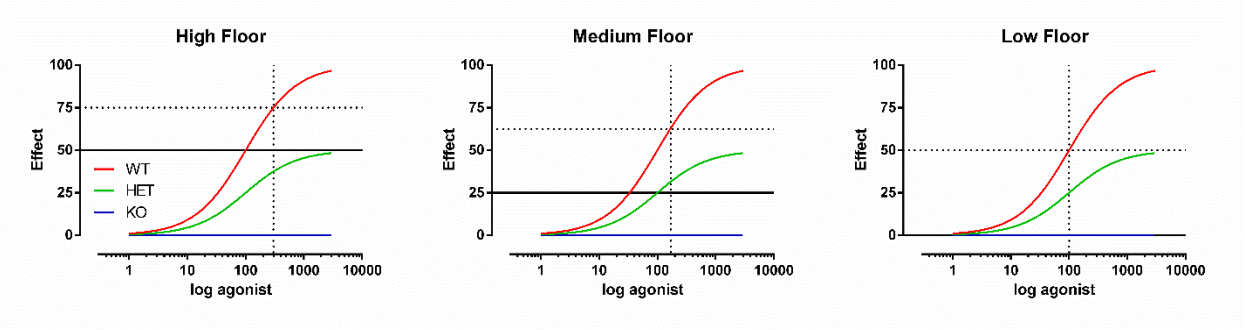

Figure 10. The experimental floor (as indicated by the solid black line) is often defined by the sensitivity of the assay. When the floor is high (i.e. sensitivity is low) then the calculated EC_{50} may be shifted to the right. As the floor becomes lower (i.e. sensitivity is increased) the EC_{50} shifts left.

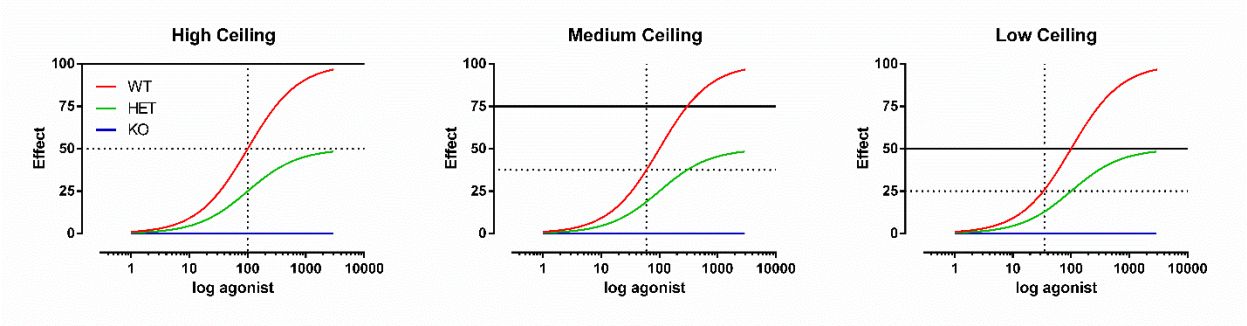

Figure 11. The ceiling (solid black line) of the assay may be the result of experimenter imposed conditions. If the ceiling is high then it could reflect the actual gain of the system, although practically it is often reduced due to outside constraints. When the ceiling is low then EC_{50} estimations are shifted left.

1.11 The cannabinoid tetrad: a summary of previous findings

As detailed above, numerous synthetic cannabinoids were synthesized utilizing a variety of backbone moieties. Binding properties of ligands to both cannabinoid receptors have been investigated using radioligand binding assays, while downstream signaling events such as G-protein signaling, cAMP accumulation, or ERK1/2 phosphorylation have also been characterized using variety of *in vitro* and *in vitro* techniques. The physiological relevance of these biochemical measurements requires translational studies. Initial cannabinoid research utilized the common dog (*Canis familiaris*) because of their considerable sensitivity to the psychoactive effects of cannabinoids. THC and other cannabinoid agonists produced ataxia (Dewey *et al*, 1972), bradycardia (Cavero *et al*, 1973), and analgesia (Kaymakçalan *et al*, 1974) in dogs. The use of canines began to fall out of favor, as reliable bioassays using mice and rats were increasingly employed. The first iteration of the cannabinoid tetrad was used to investigate structure activity relationships of THC analogs (Skinner *et al*, 1979). Later, Billy Martin and colleagues began heavy use of this method, recognizing that cannabimimetic agents produced a common spectrum of behavioral and physiological effects. specifically reduction of spontaneous activity (Martin, 1985), catalepsy, hypothermia (Beardsley *et al*, 1987), and antinociception (Martin, 1985) were utilized for these efforts. Catalepsy is defined as a fixed, rigid posture and is often measured utilizing the bar test or ring test. In the bar test, the forepaws of the subject are placed on a bar and the latency to remove themselves from the bar as set number of times (usually four) is measured. In the ring test, the animal is draped across a metal ring and latency to remove itself is measured. For both the ring test and the bar test, animals which do not have rigid posture (e.g. immobile but not cataleptic) will not remain on the apparatus. Usually the experimenter imposes a cut off time to limit the duration of the test. Antinociception can be

measured a number of ways, though the tail flick assay using radiant heat or immersion in hot water are the most common methods. In both cases, latency to remove the tail from the nociceptive stimulus is recorded. Stimulus intensity can be manipulated by adjusting the temperature, and the maximum may max also be altered though animal welfare concerns usually determine the ceiling.

This constellation of effects was eventually coined the cannabinoid "tetrad" and employed to explore *in vivo* structure activity relationships (Little *et al*, 1988b). Little and Martin also employed the stereoselective effects of cannabinoids (Little *et al*, 1989), corroborating the *in vitro* work, which satisfied the requirements for the existence of a distinct cannabinoid receptor (Devane *et al*, 1988). Subsequent work featuring the CB_1 antagonists rimonabant (Compton and Martin, 1996; Rinaldi-Carmona, 1994; Rinaldi-Carmona *et al*, 1995) or AM251 (McMahon and Koek, 2007), CB_1 KO mice (Ledent *et al*, 1999; Zimmer *et al*, 1999a) further verified the tetrad as a reliable screen for *in vivo* cannabimimetic activity. If a given ligand exhibited these four effects, it was likely to be a cannabinoid, especially when complemented by biochemical assays to determine both receptor binding and activation (Compton *et al*, 1993). A later study adapted the cannabinoid tetrad assay into a cumulative dosing procedure (Falenski *et al*, 2010) to afford a more expeditious manner of generating dose-effect relationships. Because repeated exposure to a test apparatus leads to acclimation and reduction in exploratory motor behavior, the hypolocomotion measure was eschewed.

1.12 CB_1 transgenic mice as a model: advantages and limitations

Initially cloned in 1990, the CB_1 receptor gene *Cnr1* encodes a 473 amino acid sequence contained in a single large exon (Matsuda *et al*, 1990), and is a seven transmembrane G-protein

coupled receptor, which signals via $G\alpha_{i/o}$ to inhibit downstream adenylyl cyclase (Devane *et al*, 1988). The creation of CB_1 knockout mice represented a complementary tool to CB_1 antagonists in discerning whether this receptor mediated the pharmacological effects of cannabinoids and to investigate function of endogenous cannabinoids. Three varieties of mice with constitutive deletion of CB_1 mice exist: outbred CD1 strain (Ledent *et al*, 1999), the inbred C57BL6/J (Zimmer *et al*, 1999a), and another on the C57BL6/J (Robbe *et al*, 2002). Both the Ledent and Manzoni approaches replaced a large portion of the gene as well as the upstream promoter region with a neo cassette. The Zimmer line did not replace the promoter, but rather replaced all but the first 32 and flanking 24 amino acids. Regardless, none of the lines exhibit CB_1 radioligand binding and the vast majority of putative cannabinoid ligands do not stimulate G-proteins in these KO mice. The Zimmer line is used by the vast majority of laboratories employing CB_1 transgenic mice, although a fourth, doxycycline-inducible CB_1 line exists (Marsicano *et al*, 2002), affording temporal control of CB_1 expression.

CB_1 KO mice display stark physiological and behavioral phenotypes compared to their WT and HET siblings. *In vivo*, they display reduced bodyweight, higher mortality rates, reduced locomotor activity, and a higher incidence of seizures (Zimmer *et al*, 1999a). Physiologically, they display progressive loss of hippocampal neurons with age, though adolescent CB_1 KO mice will sometimes outperform their WT littermates in the rotorod task (Bilkei-Gorzo *et al*, 2005), and increased expression of dopamine D2 receptors conveys a resistance to ethanol-induced conditioned place preference (Houchi *et al*, 2005) suggesting the deletion of CB_1 may not be entirely deleterious. This finding could be specific to ethanol, although it may reflect a more general hyposensitivity to reward as CB_1 KO imbibe lower totals of a sweetened saccharine solution (Sanchis-Segura *et al*, 2004) and do not acquire cocaine self-administration as readily as

their WT counterparts (Soria *et al*, 2005). Curiously, basal activity of delta, kappa, and mu opioid receptors seems to be elevated in CB_1 KO mice relative to WT mice (Urigüen *et al*, 2005), expression and function of $GABA_A$ and $GABA_B$ receptors is altered (Urigüen *et al*, 2011), and hypofunction of serotonergic receptors (Mato *et al*, 2007), though the behavioral ramifications of these phenotypes are not yet explored.

CB_1 KO mice are generally difficult to breed. CB_1 KO dams often display poor blastocyst implantation due to deficiencies in AEA-CB_1 signaling (Wang *et al*, 2003). CB_1 also plays a role in healthy development of sperm, as sperm from CB_1 deficient mice show impaired motility (Maccarrone, 2005). Combined, these consequences of CB_1 deletion render KO-KO breeding pairs generally unsuccessful, but the use of HET mice presents its own set of complications. During breeding, HET to HET sires and dams often do not yield a Mendelian (1:2:1 WT:HET:KO) distribution of offspring, with the knockouts often underrepresented among their littermates. Some evidence exists for pre-implantation dynamic regulation of CB_1 mRNA and endocannabinoid signaling (Paria *et al*, 1995), as well as impaired oviductal transport in CB_1 KO embryos (Wang *et al*, 2004) which may contribute to the observed, unexpected offspring distribution. This inevitably necessitates the use of both male and female mice in some cases when maintenance of large numbers of breeding HET pairs is impractical.

The use of both male and female mice introduces potential variability given known interactions of the endocannabinoid system and sex-linked characteristics and hormones. Female rats display dynamic changes in CB_1 expression during the estrous cycle, ovarectemized (OVX) rats express less CB_1 in the hypothalamus, and progesterone may rescue this deficit (Rodríguez de Fonseca *et al*, 1994). A similar study corroborated the reduced CB_1 density in the hypothalamus of OVX and male Sprague-Dawley rats, and also reported increases in amygdalar

expression of CB_1 in OVX and male rats (Castelli *et al*, 2014; Riebe *et al*, 2010). Fluctuations in a variety of endocannabinoids and related lipids also occur in tandem with estrous (Bradshaw, 2006) potentially affecting the exogenous administration of cannabinoids. Stressors may also induce changes in CB_1 expression in a sex-dependent manner, though a daily non-contingent shock paradigm may be a severe example (Xing *et al*, 2011). The potency of some cannabinoids including THC show sex-dependent differences in producing antinociception, hypolocomotion (Tseng and Craft, 2001), anti-allodynic effects, and reduction of edema (Craft *et al*, 2013) in Sprague-Dawley rats. On the other hand, the synthetic agonist CP55,940 failed to elicit significant differences in potency to increase the tail withdrawal response in adolescent Sprague-Dawley male and female rats (Romero *et al*, 2002), and THC produced few sex-related differences when injected via the intracerebroventricular route of administration (Wakley and Craft, 2011). In Long-Evans rats, THC elicited catalepsy, antinociception, hypothermia, and hypolocomotion irrespective of sex (Wiley *et al*, 2007). Agonist-stimulated [^{35}S]GTPγS of male and female rats treated chronically with THC revealed greater desensitization in female rats relative to males following application of CP55,940 (Burston *et al*, 2010).

In mice, the pattern of results is similarly mixed. Following acute treatment with THC, female mice from the outbred Swiss-Webster strain displayed both locomotor enhancement at low doses (3-10 mg/kg), and suppression at the highest dose tested (30 mg/kg) whereas males displayed exclusively suppression (Wiley, 2003b). No locomotor enhancement was detected in mice bred for wheel running behavior following WIN55,212-2 (0.5-3.0 mg/kg) administration, though females seemed resistant to the locomotor suppressing effects of the highest dose tested relative to males (Keeney *et al*, 2012)

The pharmacokinetics may also differ between male and female rodents, depending upon the drug. Both THC and its bioactive metabolite 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC) are detected in higher concentrations in the female rat brain than in male brains following an acute administration, although differences in magnitude to produce antinociception and catalepsy for either sex were not directly compared (Tseng and Craft, 2001; Wiley and Burston, 2014). In addition to pharmacokinetic differences, sex may influence the downstream signaling events which CB_1 receptors modulate. In female, but not male, guinea pig hypothalamic neurons, WIN55,212-2 increased the necessary voltage to activate A-type potassium channels (Tang *et al*, 2005), and female Listar Hooded rats display larger depolarization-induced suppression of inhibition (DSI) compared to their male counterparts (Melis *et al*, 2013).

The numerous studies highlighting the potential for sex differences when testing cannabimimetic agents collectively indicate that researchers should remain sensitive to these outcomes. Transgenic mice are often in short supply necessitating mixed sex groups of mice during experiments. While utilizing both sexes may not result in measurable differences between them, this approach also has the advantage of being potentially sensitive to these outcomes should they arise. Moreover, humans display sex-related cannabinoid effects including frequency of use, anxiety, mental health status, and pharmacokinetic differences (Fattore and Fratta, 2010), thus highlighting the importance of sensitivity to these effects via inclusion of both sexes in preclinical assays of cannabimimetic effects.

1.13 Experimental design to determine efficacy

To determine *in vivo* efficacy at the CB_1 receptor, CB_1 WT, HET, and KO mice were cumulatively dosed (similar to Falenski et al., 2010) with cannabinoid agonists until a pre-

determined maximum has been reached in for catalepsy (60 seconds of immobility), antinociception (10 seconds in a 52°C water bath), and hypothermia (Δ −8°C from baseline). The use of cumulative dosing does, however, preclude the use of hypolocomotion as measured by spontaneous activity. Observed reductions in spontaneous activity due to habituation are difficult to distinguish from CB_1-mediated hypolocomotion during repeated testing (Drew and Miller, 1973). Dose ratios calculated between WT and HET dose-effect curves via linear regression will serve as a proxy measure for efficacy differences which are not observable under these conditions due to extenuating factors influencing the ceiling of the assay. KO mice will serve as controls for potential off target effects of drugs. In cases where the solubility limits of a given compound preclude observing maximum effects, potency ratios will not be calculated although this pattern of effects provides strong evidence that the tested ligand possesses low efficacy. An alternative approach may be to consider the maximum effect stimulated by the ligands in HET mice, although solubility of the compounds at extremely high doses rendered interpretation of the data in this manner difficult. Finally, selection of ligands should include ligands of both very high and very low efficacy as determined by previously published results.

In addition to relinquishing the hypolocomotion measure, cumulative dosing versus dosing distinct groups presents a number of methodological differences and practical considerations. Practically, cumulative dosing affords the use of a much smaller number of mice, can be completed at a much faster pace, and it allows the use of repeated measures statistics. Separate dosing requires many more mice and as such is much more time consuming. One must also use between subject experimental designs which may necessitate the use of a higher number of experimental animals per group to detect statistically significant differences. Additionally, with repeated testing of the same mice one can look at the relative differences between doses in

addition to change from baseline for each individual mouse therefore permitting normalization at each data observation for each mouse rather than a before and after approach often used with separate dosing procedures. Cumulative dosing was employed here as it allowed a high throughput, within subject approach to determine dose-effect relationships with reduced number of mice compared to separate groups of animals used to determine dose-effect relationships.

Cumulative dosing is associated with potential pitfalls, notably pharmacokinetic and pharmacodynamics considerations, which are more adequately controlled in separate dosing procedures. Rodents dosed cumulatively are exposed for a much longer duration of time to drug and are often dosed at a specific interval (every 40 minutes typically) irrespective of the optimal onset and duration of the test drug. If the pretreatment time is too short or too long then maximal effects of a given dose may be missed or observed later in the presence of additional drug. This may result in an erroneous estimation of the ED_{50}. Separate dosing necessarily avoids this complication, as each animal is given the same pretreatment time for a given dose of drug. Additionally, time course studies may readily be incorporated into separate dosing experiments so that the peak effects at each dose are observed, informing the timing of dosing in subsequent experiments. In the case of cannabinoids, the duration of effects in the tetrad are typically quite long, even in mice, so in many cases a 30 minute pretreatment is likely sufficient (see Chapter 2, Figures 17-23). However, the characteristic long duration of action also introduces potential pharmacodynamic confounds to estimation of the ED_{50} and therefore potency. Tachyphylaxis, or rapid changes in the response to a drug, is a form of short term adaptation. The mechanisms underlying tachyphylaxis are not well understood in relation to cannabinoids, but on longer timescales receptor level adaptations occur. Repeated administration of numerous cannabinoids produce tolerance and cross tolerance to the acute effects of these drugs in the tetrad (Fan *et al*,

1994), and concurrent internalization, downregulation, and desensitization (Nguyen *et al*, 2012) likely occur. If an animal is exposed to progressively higher doses of an agonist capable of producing these adaptations, the likelihood that these same changes occur within session may correspond with the current cumulative dose. Thus, as increasing concentrations are administered the number of receptors available will be reduced and the remaining receptors may be less sensitive to the application of the same agonist. If tachyphylaxis occurs under cumulative dosing conditions the ED_{50} would be underestimated when compared to separate dosing. These stipulations do not consider differences among test ligands in regards to efficacy which can cause differential degrees of receptor adaptation.

The Stephenson model of receptor theory predicts that lower efficacy drugs must occupy a higher fraction of the available receptors to elicit the same response. In mice continuously infused with ED_{50} doses of μ opioid agonists which differ in efficacy, lower efficacy ligands produced the greatest degree of tolerance while much smaller magnitudes of tolerance were observed for those ligands with high efficacy (Madia *et al*, 2009). As low efficacy ligands must occupy more receptors than high efficacy ligands to elicit the same response, receptor occupancy may drive tolerance. When naloxone was administered at the peak effect of morphine administered intravenously (Hovav and Weinstock, 1987), diminished or no tolerance occurred, implicating receptor occupancy as a potential determinant of tolerance. Many cannabinoid agonists induce downregulation and desensitization *in vitro*, such as THC (Sim *et al*, 1996a), CP55,940 (Rinaldi-Carmona *et al*, 1998), and WIN55,212-2 (Jin *et al*, 1999). THC has been reported to cause considerable desensitization measured at the G-protein level while not appreciably reducing receptor number in some brain regions such as cerebellum, hippocampus, and striatal regions (Breivogel *et al*, 1999), perhaps indicating higher efficacy ligands reduce

receptor sensitivity to subsequent stimulation while occupying few receptors resulting in minimal downregulation. Another study using WIN55,212-2 and THC in which ED_{50} doses were doubled every three days revealed that this may not be correct, as each drug caused similar reductions in CB_1 receptors across brain regions while THC produced much higher levels of desensitization. Additionally, chronic administration of the low efficacy endocannabinoid AEA (Breivogel *et al*, 2001; Griffin *et al*, 1998) in rats does not result in downregulation while substantial desensitization occurs (Rubino *et al*, 2000). This study does not however, consider the rapid metabolism of AEA by fatty acid amide hydrolase (FAAH) (Giang and Cravatt, 1997), which would effectively render AEA concentration in the brain negligible. Additionally, AEA is known to produce agonist-stimulated [^{35}S]GTPγS binding in mice devoid of CB_1 (Di Marzo *et al*, 2000); thus, a non-CB_1 mechanism likely accounts for the observed desensitization. Though evidence for receptor occupancy driving cannabinoid tolerance remains inconclusive, it nonetheless should be considered in terms of cumulative dosing when utilizing mice which already possess a reduced number of receptors as is the case with CB_1 HET mice. A high efficacy ligand would require fewer receptors to elicit the same response as a lower efficacy ligand, therefore a higher fractional occupancy is necessary. The degree of tachyphylaxis may be higher for drugs which require higher numbers of receptors, therefore the ED_{50} estimation under cumulative dosing conditions might shift further to the right for lower efficacy drugs as compared to the same drug tested with separate dosing. When trying to make comparisons between drugs to assess efficacy with cumulative dosing, the apparent ED_{50} for higher efficacy drugs is likely closer to that of separate dosing groups while lower efficacy drugs may be shifted considerably to the right. This effect means that resolution to differentiate CB_1 ligands which possess high efficacy would be diminished while lower efficacy drugs may more readily be

differentiated. Utilizing experimental endpoints which depend upon CNS regions possessing lower concentrations of CB_1 would potentially exacerbate this effect, while very high concentrations of CB_1 could functionally negate this effect.

The CB_1 ligands tested were chosen based upon previously published results in both triad/tetrad experiments as well as agonist-stimulated [^{35}S]GTPγS binding. Most cannabimimetic agents elicit full suppression of spontaneous locomotor activity, up to several minutes of catalepsy in the ring immobility (Wiley *et al*, 1998) and bar tests (Falenski *et al*, 2010), significant reductions in body temperature, and increases in latency to withdrawal responses from a noxious stimuli. However, considerable methodological differences exist across the literature for each endpoint and in most cases the ceiling of the assay is determined arbitrarily. Agonist-stimulated [^{35}S]GTPγS binding provides an enhanced ability to distinguish efficacy between ligands via measuring the functional activity at the CB_1 receptor as one may readily measure difference in maximal activation, (E_{max}). The differentiation in efficacy depends on a number of factors which are discussed more specifically above, but it should be noted that GDP-GTP exchange at the G-protein does not fully encompass the spectrum of effects elicited by cannabinoids via the CB_1 receptor. Many other pathways are possible including BARR2-mediated ERK1/2 signaling, which may interfere with the *a priori* selection of cannabinoids based upon their efficacy to produce effects at one level for one pathway. As such, it will be necessary to compare findings in the triad to functional CB_1 activity via agonist-stimulated [^{35}S]GTPγS binding.

1.14 Cannabinoids in preclinical abuse liability testing assays

Homo sapiens have imbibed marijuana in various forms for millennia, both for therapeutic and recreational uses. The intoxicating effects are well documented and elicited

primarily by THC through the activation of CB_1 receptors (Huestis *et al*, 2001), and marijuana remains the most popular drug of abuse worldwide. CB_1 receptors are expressed on GABAergic and glutamatergic neurons (Tsou *et al*, 1998) and are abundant in reward-relevant regions of the mammalian brain such as the ventral tegmental area (VTA) and the nucleus accumbens (NAcc) (Herkenham *et al*, 1990). Relatively low doses of THC evoke dopamine release in the NAcc (Chen *et al*, 1991; Ng Cheong Ton *et al*, 1988), which is thought to be a common mechanism for many drugs of abuse (Di Chiara and Imperato, 1988). Despite its abundant use by man, THC remains an enigma in preclinical abuse liability testing, as it fails to elicit reliable abuse-related effects in the many assays of abuse liability, including conditioned place preference (CPP), self-administration (SA), and intracranial self-stimulation (ICSS).

CPP is a Pavlovian conditioning procedure in which animals (typically mice or rats) are exposed to two chambers with a variety of differing cues including the pattern on the walls, the floor texture, and potentially other cues. A neutral connecting chamber is sometimes present as well. A drug is then randomly paired for each subject to one of the chambers while an appropriate vehicle is paired to the other. The subject is later returned to the apparatus with access to all chambers and the time it spends in each is counted. Common drugs of abuse such as psychomotor stimulants (Spyraki *et al*, 1982), opiates (Mucha *et al*, 1982), ethanol (Shimizu *et al*, 2015), and nicotine (Kota *et al*, 2008) produce condition place preference wherein the subject spends more time in the drug paired side than it did prior to conditioning (or more time in the drug-paired versus vehicle-paired chamber), whereas aversive drugs, such as kappa opioid receptor agonists (Suzuki *et al*, 1992) or lithium chloride (Mucha *et al*, 1982), elicit conditioned place aversion such that the subject spends less time in the drug paired side. THC has been reported to elicit conditioned place preference (Braida *et al*, 2004; Lepore *et al*, 1995; Manwell

et al; Valjent *et al*, 2002) as well as place aversion (Cheer *et al*, 2000; Manwell *et al*; Parker and Gillies, 1995; Sañudo-Peña *et al*, 1997). Similarly, SCs, such as CP55,940 (McGregor *et al*, 1996), WIN55,212-2 (Chaperon *et al*, 1998), and HU-210 (Cheer *et al*, 2000), tend to elicit conditioned place aversion though a conditioned place preference has been reported for very low doses of CP55,940 (Braida *et al*, 2001a). Pre-exposure to the test ligand in the absence of the chamber may unmask a conditioned place preference for THC (Valjent and Maldonado, 2000) and the synthetic cannabinoid AM281 (Botanas *et al*, 2015), although the underlying neurobiological mechanisms are not well understood.

SA has long stood as the standard for testing the abuse liability of drugs. Laboratory animal models of SA are generally highly predictive of abuse potential in humans. While abused drug classes, such as opiates and psychomotor stimulants, are readily self-administered (van Ree *et al*, 1978), cannabinoid SA has been difficult to demonstrate in laboratory animals. Rhesus monkeys failed to acquire THC self-administration under fixed-interval schedule (Mansbach *et al*, 1994), while squirrel monkeys self-administered very low doses of THC (Justinova *et al*, 2003; Tanda *et al*, 2000). Intracerebroventricular self-administration of THC was also achieved in Wistar rats (Braida *et al*, 2004). SA of SCs is also difficult to demonstrate in laboratory animals, though WIN55,212-2 SA seems comparatively robust relative to that of other cannabinoids. WIN55,212-2 is self-administered by DBA mice at very low doses (Martellotta *et al*, 1998) and by Long-Evans rats at low doses as well under an FR1 schedule (Fattore *et al*, 2001). CD1 mice also self-administer WIN55,212-2 if given a priming dose (Mendizábal *et al*, 2005). Sprague-Dawley rats trained to self-administer WIN55,212-2show an elevation of extracellular dopamine in the NAcc shell, but not in the core (Lecca *et al*, 2006). This effect was also found in Listar Hooded and Long-Evans rats (Fadda *et al*, 2006). In a subsequent study by

the same research group, Lister Hooded and Long-Evans rats, but not Sprague-Dawley rats, self-administered WIN55,212-2 (Deiana *et al*, 2007). In another study, Long-Evans rats learned to self-administer WIN55,212-2, but failed to acquire THC self-administration, calling into question the validity of WIN55,212-2 as a screen for cannabinoid abuse liability (Lefever *et al*, 2014). Other cannabinoid agonists are also self-administered to some degree, though reports are scarce. Wistar rats self-administered CP55,940 via the intracerebroventricular route of administration (Braida *et al*, 2001b) and the endogenous cannabinoid 2-AG was self-administered by squirrel monkeys (Justinová *et al*, 2011). Finally, JWH-018 was self-administered by both Sprague-Dawley and C57BL/6J mice and increases extracellular dopamine in rats (De Luca *et al*, 2015). To date, it is unknown why some SCs, and WIN55,212-2 in particular, are readily self-administered by some strains while THC is generally not regardless of strain. This effect may be related to the partial agonist properties of THC, or the apparently very narrow dose range at which THC might be self-administered. Higher doses of THC produce aversive-like states in other assays such as CPP thus SA of THC may also elicit a similarly aversive state.

ICSS is another preclinical assay of abuse liability which has good predictive validity for drugs of abuse (Negus and Miller, 2014). ICSS functions via implantation of an electrode into the medial forebrain bundle (MFB) (Olds & Milner, 1954), a tract of ascending and descending neuronal projections, some of which synapse onto dopaminergic neurons in the VTA, which then project into the NAcc resulting in release of dopamine in the NAcc (Phillips *et al*, 1989; Stellar and Stellar, 1985). The results of tandem ICSS and fast scan cyclic voltammetry (Kruk *et al*, 1998) and microdialysis studies (Miliaressis *et al*, 1991) suggest that dopamine release alone may not be sufficient to drive the behavior, though strong neurochemical evidence implicates

dopamine release as a major component of ICSS (Fiorino *et al*, 1993). Electrical stimulation of the MFB elicits vigorous operant responses for brain stimulation. The electrical stimulation offers tight temporal control and the frequency and amplitude of the electrical stimulation can be easily manipulated within session to generate a wide range of response rates in a relatively short period of time. Facilitation of ICSS is considered predictive of abuse liability, while suppression may indicate abuse-limiting effects (Negus and Miller, 2014). Monoamine releasers (Bauer *et al*, 2013), mu opioid agonists (Altarifi *et al*, 2013), $GABA_A$ agonists (Tracy *et al*, 2014), and nicotinic agonists (Freitas *et al*, 2015) facilitate ICSS. In contrast, kappa opioid agonists (Negus *et al*, 2010) and serotonin releasers (Bauer *et al*, 2015) tend to suppress ICSS. THC has been reported to modestly facilitate ICSS in Lewis rats at a dose of 1.5 mg/kg (Gardner *et al*, 1988) and Sprague-Dawley rats at doses of 1.0 mg/kg and 0.1 mg/kg (Katsidoni *et al*, 2013; Lepore *et al*, 1996), though other experiments indicate it produces little effect at low doses and suppression of ICSS at high doses in Sprague-Dawley rats (Kwilasz and Negus, 2012; Vlachou *et al*, 2007) and C57BL/6J mice (Wiebelhaus *et al*, 2015). In contrast to its effects in self-administration studies, WIN55,212-2 produces marked suppression of ICSS in Sprague-Dawley rats (Mavrikaki *et al*, 2010; Vlachou *et al*, 2004). CP55,940 suppresses ICSS in Sprague-Dawley rats (Arnold *et al*, 2001; Kwilasz and Negus, 2012; Vlachou *et al*, 2004) and in C57BL/6J mice (Grim *et al*, 2015). HU-210 (Vlachou *et al*, 2004) suppresses ICSS in Sprague-Dawley rats. Inhibition of FAAH, the primary catabolic enzyme of anandamide, does not affect ICSS at low doses but produces a small suppression of ICSS via a non-CB_1, non-CB_2 mechanism (Kwilasz and Negus, 2012; Wiebelhaus *et al*, 2015). Similarly, inhibition of monoacylglycerol lipase (MAGL), which elevates endogenous 2-AG, suppresses ICSS in a CB_1-dependent manner (Wiebelhaus *et al*, 2015).

Although cannabinoids do not display the usual pattern of results that most other drugs of abuse display, the fact remains that humans abuse them. This discrepancy may reflect a failure to identify assay conditions in which abuse-related properties are unveiled. For instance, blood concentrations of THC in humans smoking marijuana are quite low (~10 ng/ml peak) (Cone *et al*, 2015) compared to blood concentrations achieved via interperitoneal injection necessary to produce robust catalepsy, hypothermia, and antinociception in mice (~500-1000 ng/ml) (Falenski *et al*, 2010), reflecting a large gap between the doses that are self-administered in humans versus the experimentally-induced doses in preclinical assays. Route and method of administration may also play a role. Animal models generally rely upon non-contingent, intravenous or interperitoneal injection as opposed the human method of ingestion which is generally smoked and done so purposefully. Ongoing efforts to identify the abuse liability of cannabinoids nonetheless remain important, and alternative approaches are clearly needed. Testing for tolerance and dependence represents a possible mechanism by which other aspects of drug abuse beyond the acute abuse-related effects could be assessed.

1.15 Dependence and synthetic cannabinoids in humans and laboratory animals

Abrupt cessation of marijuana consumption following prolonged use can lead to withdrawal in humans. Cannabinoid withdrawal features at least three of the following seven clinical signs: 1) irritability, anger, or aggression; 2) nervousness or anxiety; 3) sleep difficulty (e.g. insomnia, disturbing dreams); 4) decreased appetite or weight loss; 5) restlessness; 6) depressed mood; and 7) physical symptoms such as abdominal pain, shakiness, tremors, sweating, fever, chills, or headache (American Psychiatric Association, 2013). Heavy cannabis users report experiencing robust withdrawal symptoms following discontinuation of cannabis use

(Allsop *et al*, 2012). Laboratory animal models of cannabinoid dependence have been developed in which a cannabinoid is administered over a period of days, and then subjects are challenged with a CB_1 receptor antagonist to precipitate withdrawal. The most common precipitated somatic withdrawal signs include paw tremors and head shakes, and are readily observed following rimonabant challenge in THC-dependent rats (Aceto *et al*, 1995, 1996) and mice (Cook *et al*, 1998). Rimonabant is known to act as an inverse agonist (Smith *et al*, 2015), suggesting that the ensuing withdrawal signs in cannabinoid-treated animals is the result of this property, instead of simple displacement of the agonist. However, recent work by Jarbe's group demonstrated that the neutral CB_1 receptor antagonist AM4113, precipitated withdrawal responses in THC-dependent animals, demonstrating that CB_1 receptor blockade is sufficient to precipitate withdrawal (Tai *et al*, 2015b). In contrast to precipitated withdrawal models, spontaneous withdrawal from cannabinoids is more difficult to detect, though spontaneous somatic signs of withdrawal were detected following continuous infusion of WIN55,212-2 (Aceto *et al*, 2001).

Non-somatic signs of withdrawal have been more difficult to demonstrate, even when withdrawal is precipitated. Mice treated with THC sub-chronically and administered rimonabant displayed deficits in spatial memory as measured by the Morris water maze (Wise *et al*, 2011). Rimonabant precipitated withdrawal also unveils an anxiety-like phenotype in mice (Huang *et al*, 2010). Abrupt cessation of CP55,940 (0.5 mg/kg, b.i.d., 7 days) produced an anxiety-like phenotype and locomotor depression in rats (Aracil-Fernández *et al*, 2013), while cessation of HU-210 elicited only hypolocomotion (Moreno *et al*, 2005). To date, only two studies investigated the consequences of repeated cannabinoid administration on reward processes utilizing the ICSS procedure (Chapter 3) (Grim *et al*, 2015; Mavrikaki *et al*, 2010). In the case of WIN55,212-2, rats did not become tolerant to the rate decreasing effects of WIN55,212-2,

despite 22 days of administration, and no mention of withdrawal from these effects was reported. Similarly, mice treated repeatedly with CP55,940 did not display signs of spontaneous or precipitated withdrawal following seven days of dosing, though tolerance was reported (Chapter 3) (Grim et al., 2015).

1.16 Objectives and hypotheses

Given the dissimilar patterns of abuse-related and toxicological effects between THC and SCs, there is a need to assess potential mechanisms that might mediate the obvious discrepancy. SCs also generally possess unknown pharmacology and toxicology, and they are generally used first in man, highlighting the need for *in vivo* methods of assessing their pharmacological properties. One aspect of abused SCs which distinguishes them from THC is their enhanced potency and efficacy at the CB_1 receptor. A first step to determine whether or not this enhanced CB_1 activity plays a role in the health complications engendered by SCs is determination of their relative efficacies *in vivo*, and what effect that may have upon abuse-related behaviors. The research presented in this thesis investigated the consequences of acute and repeated administration of synthetic cannabinoids in established mouse models of abuse and cannabimimetic activity.

As SCs possess a spectrum of effects ranging from toxicity to abuse-related effects, they should be assessed in assays that capture effects on various physiological effects and effects on reward processes. Here, I utilized a "bottom-up" approach, first characterizing the efficacy of six cannabinoids for their CB_1 functional activity in an *in vitro* assay and centrally-mediated physiological consequences of CB_1 activation in the whole animal. Next, one ligand (CP55,940) was selected as a representative SC and assessed for abuse-related effects on reward processes. More specifically, I applied basic pharmacological principles to determine *in vitro* and *in vivo*

efficacy and receptor mediation of synthetic cannabinoids (see Chapter 2) and then assessed the abuse- and dependence-related properties of the synthetic cannabinoid CP55,940 in the ICSS (see Chapter 3). Together, these aims will explore the contribution of efficacy to elucidate possible differences between THC and abused SCs.

Hypothesis: SCs will have enhanced efficacy at the CB_1 receptor as compared to THC to elicit in vivo cannabimimetic effects, and this will translate into enhanced abuse-related effects.

1.17 Chapter 2: hypothesis

Determination of *in vivo* efficacy and receptor mediation of cannabimimetic effects were conducted using CB_1 WT, HET, and KO mice in a cumulative dosing procedure in which mice were assessed for catalepsy, hypothermia, and antinociception (the cannabinoid triad) after each dose The cannabinoid triad was utilized to establish a rapid screening method to ascertain *in vivo* efficacy as well as test whether a 50% reduction in CB_1 receptors will produce a concordant loss of potency in the CB_1 HET mice. Additionally, CB_1 KO mice enabled the detection of whether any of the test compounds elicited non-CB1 triad effects. ED_{50} values were calculated by linear regression after conversion to % effect, when appropriate, to examine shifts in potency between CB_1 WT and HET mice. The ceiling of each measure is as follows: 60 seconds for catalepsy, -8°C from baseline for hypothermia, and 10 seconds for tail withdrawal. Data transformations are detailed below. Dose ratios will be calculated by dividing the WT ED_{50} by the HET ED_{50}.

$$\text{Catalepsy} = \frac{test}{60} \text{ x } 100$$

$$\text{Hypothermia} = \frac{test - baseline}{-8} \text{ x } 100$$

$$\text{Antinociception} = \frac{test - baseline}{10 - baseline} \times 100$$

Hypothesis: For the *in vivo* determination of efficacy, the efficacy of the test ligand is expected to vary inversely with the potency shift observed between WT and HET mice, while KO display no pharmacological effects of each agonist. Furthermore, this potency shift will correlate with *ex vivo* agonist stimulated [35S]GTPgS binding utilizing the same test drugs in WT, HET, and KO CNS tissue. The non-CB1 drugs morphine and chlorpromazine are expected to be equipotent across WT, HET, and KO mice.

1.18. Chapter 3: hypothesis

For abuse liability and dependence testing in ICSS, CP55,940 was selected as an archetypal representative of highly potent and efficacious synthetic cannabinoids commonly found in abused preparations. To assess whether synthetic cannabinoids facilitate ICSS an acute dose response of CP55,940 will be determined. To determine whether rate decreasing or rate increasing effects are CB_1-mediated, a high dose of CP55,940 will be preceded by a dose of rimonabant sufficient to return responding to baseline levels. Tolerance to these effects will be assessed over seven days of dosing with a high dose of CP55,940 after which changes in baseline responding will be analyzed for changes related to spontaneous withdrawal. The same seven day dosing procedure will be used again, this time with a high dose of rimonabant on the final day to assess precipitated withdrawal. Together, these studies will examine the effect of both acute and repeated administration of a highly potent and efficacy cannabinoid on brain reward processes.

Hypothesis: For abuse-liability and dependence testing utilizing the representative SC CP55,940 in ICSS, mice will display changes in rates of responding for ICSS following spontaneous and

precipitated withdrawal. Acutely, CP55,940 will suppress rates of responding in a CB_1 dependent manner, and rimonabant will elicit no rate-suppressing effects.

Chapter 2

Stratification of cannabinoid 1 receptor (CB_1) agonist efficacy: Manipulation of CB_1 density through use of transgenic mice reveals congruence between *in vivo* and *in vitro* assays

Grim TW[1], Morales AJ[1], Gonek MM[1], Wiley JL[2], Thomas BF[2], Sim-Selley LJ[1], Selley DE[1], Negus SS[1], Lichtman AH[1].

Virginia Commonwealth University – Pharmacology and Toxicology, Richmond, VA, USA[1]

Research Triangle Institute, Winston-Salem, NC, USA[2]

Manuscript in preparation for submission to Neuropsychopharmacology.

ABSTRACT

Diversion of synthetic cannabinoids (SCs) from research purposes to abused preparations threatens public health. Ensuing emergency scheduling resulted in clandestine synthesis of novel SCs with heretofore unseen structures detected in abused preparations. Here, we incorporated commonly employed *in vivo* and *in vitro* assays, CB_1 transgenic (CB_1 wild type (WT), heterozygous (HET), and knockout (KO)) mice to vary receptor density, and basic pharmacological principles to provide insight into the potency, selectivity and efficacy of CB_1 receptor-mediated effects produced by these rapidly emerging drugs of abuse. Accordingly, we examined the dose-response relationships of THC and five SCs in producing well-established *in vivo* (catalepsy, hypothermia, and antinociception) and *in vitro* (agonist-stimulated GTPγS binding) pharmacological effects of cannabinoids. Whereas cannabinoid-induced catalepsy was resistant to a 50% reduction in CB_1 density, hypothermia and antinociception showed ligand-dependent increases in agonist ED_{50} values and decreases in E_{max} values. In contrast, non-cannabinoid compounds (morphine and chlorpromazine) produced pharmacological effects in subsets of these assays, regardless of genotype. *In vitro* CB_1 activity assessed by agonist-stimulated GTPγS binding significantly correlated with altered drug potency (WT ED_{50} ÷ HET ED_{50}) to produce hypothermia (r=0.84) and antinociception (r=0.95), but not catalepsy. These findings suggest that neural substrates subserving cannabinoid-induced antinociception and hypothermia contain a smaller CB_1 reserve than for cannabinoid-induced catalepsy. More generally, this study offers a conceptual framework and high-throughput screening method to evaluate potency, selectivity and efficacy of not only emerging abused cannabimimetic ligands, but also naturally-occurring cannabinoids and other cannabinoids being developed as research tools or potential therapeutics.

1. Introduction

Synthetic cannabinoids (SCs), comprised of myriad structures and largely unknown pharmacology (Kronstrand *et al*, 2013; Louis *et al*, 2014; Sobolevsky *et al*, 2015), have emerged as drugs of abuse representing significant public health threats (Law *et al*, 2015; Trecki *et al*, 2015). In stark contrast to THC, the primary psychoactive constituent of cannabis, SCs have been linked to life-threatening medical complications (Clark *et al*, 2015; Freeman *et al*, 2013; Mir *et al*, 2011; Takematsu *et al*, 2014; Thornton *et al*, 2013), psychological complications (Celofiga *et al*, 2014; Meijer *et al*, 2014; Peglow *et al*, 2012; Schwartz *et al*, 2015; Thomas *et al*, 2012), and death (Behonick *et al*, 2014; Gerostamoulos *et al*, 2015; Shanks *et al*, 2015; Westin *et al*, 2015). These clinical observations suggest that SCs pose a more general threat than cannabis/THC to public safety. Withdrawal-like symptoms from SCs have also been reported (Nacca *et al*, 2013; Sampson *et al*, 2015), indicating a possibility for dependence.

The mechanisms that underlie heightened risk for medical complications by SCs in comparison to cannabis/THC are not known and may vary according to the particular SC under consideration. Similar to THC, SCs bind and activate CB_1, a GPCR, which plays an important role in mediating behavioral effects produced by marijuana and CB_1 agonists (Huestis *et al*, 2001; Rinaldi-Carmona, 1994). Effects of SCs at non-CB_1 could be one factor that contributes to toxicity associated with these drugs (Sherpa *et al*, 2015). Moreover, based largely on results from *in vitro* assays of agonist-stimulated [^{35}S]GTPγS binding (Burkey *et al*, 1997a, 1997b; Selley *et al*, 1996), THC is defined as a low-efficacy CB_1 agonist, whereas many SCs are defined as high efficacy CB_1 agonists. Accordingly, high efficacy of SCs at CB_1 may also contribute to heightened risk for clinical complications. However, existing *in vivo* assays used to assess cannabinoid effects have poor resolution for distinguishing CB_1 agonist efficacy. For example,

although *in vitro* assays of agonist-stimulated [^{35}S]GTPγS binding indicate that THC and the SC WIN55,212-2 possess low and high efficacy at CB_1 receptors, respectively (Griffin *et al*, 1998), these drugs produce similar maximal effects in assays of catalepsy, hypothermia and antinociception that are commonly used to assess behavioral pharmacology of cannabinoids in mice (Fan *et al*, 1994). Drug discrimination procedures, in which efficacy requirements are manipulated by using different training doses of a high-efficacy cannabinoid training drug (Järbe *et al*, 2014) or by induction of tolerance to THC (Hruba *et al*, 2012), represent effective strategies to improve sensitivity of behavioral assays to detect agonist efficacy. However, throughput with these procedures is relatively slow, and they have not been widely used. Consequently, new strategies for rapid *in vivo* evaluation of CB_1 selectivity and efficacy could facilitate efforts to evaluate pharmacology of novel SCs and predict potential for abuse or clinical harm.

Here, we hypothesize that comparison of in vivo drug effects in CB_1 wild type (WT) and knockout (KO) mice will provide information on CB_1 selectivity of SCs, and comparison of drug effects in WT and CB_1 heterozygous (HET) mice will provide information on CB_1 efficacy. To test this hypothesis, we utilized CB_1 KO, HET, and WT mice to determine *in vivo* and *in vitro* effects of THC, CP55,940, WIN55,212-2, and SCs associated with abuse (CP47,497, JWH-073 (Atwood *et al*, 2011), and A-834,735D). We incorporated a similar *in vivo* approach to that used previously for investigation of mu opioid receptor (MOR) agonist efficacy, in which MOR HET mice showed diminished morphine antinociception compared with WT mice (Sora *et al*, 2001). The present study employed a cumulative dosing procedure to increase throughput in determining the dose-response relationships of each ligand to elicit well described cannabimimetic effects (i.e., catalepsy, hypothermia, and antinociception). In addition, the non-

cannabinoids morphine and chlorpromazine, which are active in some of these assays (Wiley, 2003a), were included as negative controls predicted to produce effects independent of CB_1 genotype. Finally, [^{3}H]SR141716A binding was conducted to confirm that CB_1 density was reduced by half in HET mice, and drug effects on agonist-stimulated [^{35}S]GTPγS binding were evaluated to provide an in vitro correlate for in vivo measures of CB_1 selectivity and efficacy. Membranes from both cerebellum and spinal cord from WT, HET and KO mice were used in assays of [^{35}S]GTPγS binding to provide tissue sources with varying CB_1 densities.

2. Methods

2.1 Subjects

Male and female CB_1 WT, HET, and KO mice (Zimmer *et al*, 1999a) derived from CB_1 HET breeding pairs backcrossed at least 15 generations with C57BL/6J mice served as subjects. Mice had *ad libitum* access to food and water and were maintained on a 12 h light/dark cycle. Approximately 80 mice between 8 and 36 weeks of age were used for all experiments, which were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee (IACUC).

2.2 Drugs

Studies were conducted with THC and with five SCs purported to have higher efficacy than THC at CB_1 receptors. The five SCs were (in order from purported highest to lowest efficacy) A-834-735D, WIN55,212-2, and CP55,940, JWH-073, CP47,497 (Atwood *et al*, 2011; Auwärter *et al*, 2009; Griffin *et al*, 1998). The mu opioid receptor agonist morphine and dopamine receptor antagonist chlorpromazine were also studied as negative controls expected to produce behavioral effects insensitive to the CB_1 receptor density. A-834,735D, WIN55,212-2, CP47,497, JWH-073 and chlorpromazine were obtained from Cayman Chemical (Road, Ann Arbor, MI), and morphine, THC and CP55,940 were generously supplied by the National Institutes on Drug Abuse Drug Supply Program (Research Triangle Institute, Raleigh, NC) for behavioral experiments. Each drug was dissolved in 100% ethanol, an equal volume of Emulphor EL-620 was added, and then 18 parts of 0.9% saline was added for a final ratio of 1:1:18. For binding assays, THC-CRM was acquired from Cayman, and [^{3}H]SR141716A was purchased from Perkin-Elmer (Waltham, MA).

2.3 Behavioral Assays

To assess *in vivo* cannabimimetic activity, catalepsy, hypothermia and antinociception (see Supplementary Methods for details) were measured at baseline, and cumulative dose response curves were established for each test compound as previously described (Falenski *et al*, 2010). The three tests required approximately 10 min to complete for six mice; thus groups of six mice were injected every 40 min with increasing doses of the test agonist and tested 30 min after each injection. Dose-effect curves for each agonist on each behavioral endpoint were analyzed by two-way ANOVA, with dose and genotype as the two factors. A significant ANOVA was followed by Holm-Sidak post hoc analysis, assessing dose-dependent changes within genotype as well as differences in drug effect between genotypes at each dose. In addition, ED_{50} values and 95% confidence limits for drug effects on behavioral measures were determined via linear regression (Colquhoun, 1971), and ED_{50} values were considered to differ if 95% confidence limits did not overlap. The ED_{50} was defined as the dose to produce immobility for 30 sec in the catalepsy test, a 4°C loss in body temperature in the hypothermia test, or 50% of the maximum possible effect in the antinociception test. To assess changes in agonist effects produced by the lower CB1 receptor density in HET vs. WT mice, dose ratios (DR) were calculated using the equation (WT ED_{50}/HET ED_{50}) for each agonist on each behavioral measure. We hypothesized that this dose ratio would serve as an *in vivo* measure of agonist efficacy.

2.4 Cellular Assays

2.4.1 Membrane Preparation

Male and female CB_1 WT, HET, and KO mice were euthanized via rapid decapitation. Cerebellum were harvested and bisected and spinal cords were taken from lumbar to cervical regions. Details for membrane can be found under Supplementary Methods.

2.4.2 [^{3}H]SR141716A radioligand binding

Using established methods (Selley *et al*, 2001), cerebellum and spinal cord samples were diluted with assay buffer to 10 μg/ml and 15 μg/ml, respectively. Membrane homogenates were then incubated with [^{3}H]SR141716A (0.03-10 nM) in the absence and presence of a saturating concentration of unlabeled SR141716A (5 μM) to assess specific and non-specific binding. The assay was incubated until equilibrium was attained (90 min) at 30 °C, and then the reaction was terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters pre-soaked in Tris buffer containing 0.5% bovine serum albumin (BSA) followed by three washes. Bound radioactivity was measured via liquid scintillation spectrophotometry at 45% efficiency after a 9 h delay to allow the liquid scintillation fluid to dissolve the filter paper. For [^{3}H]SR141716A radioligand binding assays, saturation binding (B_{max}) and affinity were determined by non-linear regression saturation analysis in GraphPad Prism 6.0. Data are expressed as mean values ± standard error of the mean.

2.4.3 Agonist-stimulated [^{35}S]GTPγS binding

Following membrane preparation, varying doses of cannabinoid agonist were added to 12 mm x 75 mm silicate tubes along with 30 μM guanosine diphosphate (GDP), 0.1 nM [^{35}S]GTPγS, and 0.1% bovine serum albumin in duplicate and incubated at 30° C for 2 h (Lazenka *et al*, 2015). Non-specific binding was determined in the presence of 20 μM unlabeled GTPγS. The reaction was terminated by vacuum filtration through grade GF/B glass fiber filters

followed by two washes with cold Tris buffer (50 mM, pH 7.4). After overnight extraction in scintillation fluid (Research Products International, Mount Prospect, IL), bound radioactivity was assessed via liquid scintillation spectrophotometry at 95% efficiency. In agonist-stimulated [^{35}S]GTPγS binding experiments, non-specific binding was subtracted from each drug curve, and data were expressed as % net stimulation (net stimulation / basal x 100). As a small magnitude of stimulation was detected in CB_1 KO tissue in certain instances (e.g., WIN55,212-2) (Breivogel *et al*, 2001; Monory *et al*, 2002), the stimulation from KO tissue was subtracted from CB_1 WT and HET curves to provide a clearer representation of CB_1-mediated agonist-stimulated binding. Maximal stimulation (E_{max}) and EC_{50} values were determined via nonlinear regression using GraphPad Prism 6.0 software. Significant differences between WT and HET E_{max} values were determined by Student's T-test for each drug. Differences in E_{max} across WT samples were analyzed using one-way ANOVA followed by a Tukey post hoc test.

2.5 Data Analyses

To assess whether decreases in drug potency to produce behavioral effects in CB_1 HET mice correlated with reductions in E_{max} values generated in agonist-stimulated [^{35}S]GTPγS binding assays, Pearson correlations were calculated between dose ratios (WT ED_{50} / HET ED_{50}) from behavioral experiments and WT E_{max} from *in vitro* studies. These correlations were performed using in vivo data from each behavioral assay (catalepsy, hypothermia, and antinociception) vs. in vitro data from each tissue source (cerebellum and spinal cord).

Results

3.1 i*n vivo* effects of cannabinoids in CB_1 WT, HET, and KO mice

Figure 12 depicts tail withdrawal responses of A-834,735D (Figure 12A), WIN55,212-2 (Figure 12B), CP55,940 (Figure 12C), JWH-073 (Figure 12D), CP47,497 (Figure 12E), and THC (Figure 12F) in WT, HET, and KO mice. As shown in Table 3, the antinociceptive potency of WIN 55,212-2 was similar between HET and WT mice, while the dose ratio of each of the other cannabinoids revealed decreased potency in HET mice compared with WT mice. Within the dose range tested, the maximum %MPE values for JWH-073, CP47,497, and THC were lower in HET mice than in WT mice. As the magnitude of respective effects of CP47,497 and THC did not surpass 50% and 25% MPE, neither the antinociceptive ED_{50} values in HET mice nor the dose-ratios (WT/HET mice) could be calculated.

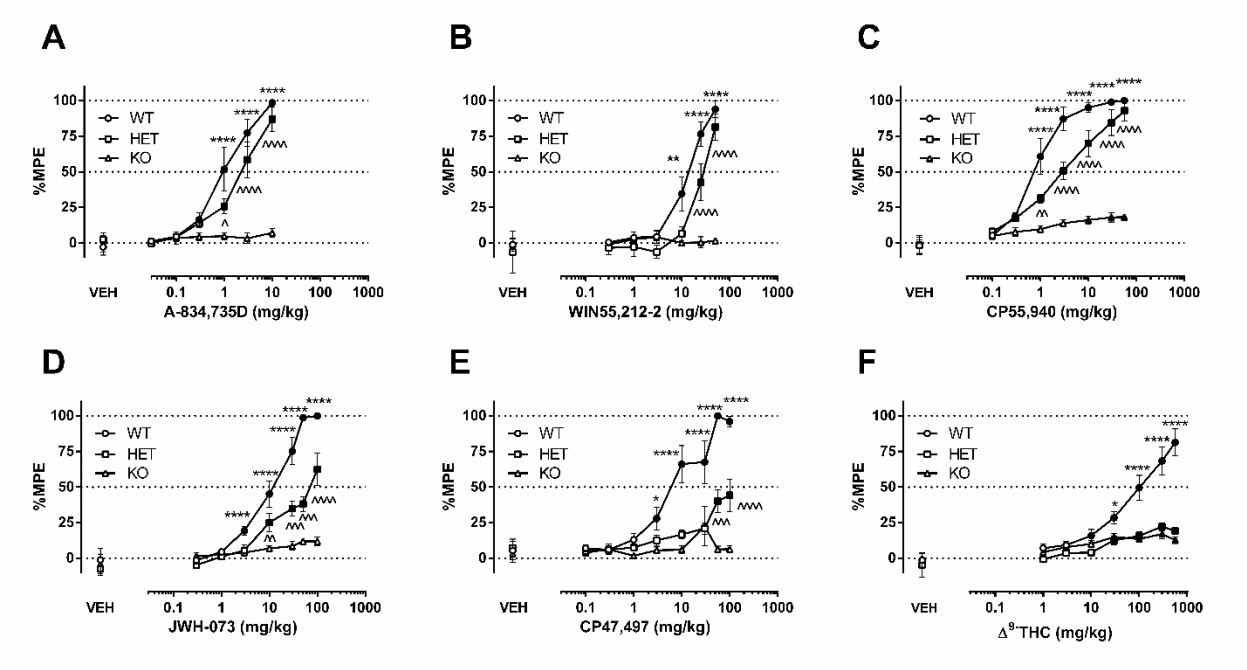

Figure 12. Dramatic differences in potencies and efficacies of THC and SCs between CB_1 WT and HET mice in producing antinociception. The high efficacy agonists A-834,735D (**A**, PR (95% CL) = 1.83 (1.11-3.11)) and WIN55,212-2 (**B**, PR (95% CL) = 2.54 (1.38-4.97)) produced comparable shifts in potency. CP55,940 (**C**, PR (95% CL) = 7.32 (3.47-18.48)) differed significantly from A-834,735D, but not from WIN55,212-2. JWH-073 (**D**), CP47,497 (**E**), and THC (**F**) failed to produce maximal effects in CB_1 HET mice, which precluded the accurate calculation of potency ratios. The mean ± SEM baseline tail withdrawal latency for all groups was 2.05 ± 0.05 s. VEH indicates an injection of 1:1:18 vehicle prior to cumulative dosing with the indicated drug. Filled shapes indicate $p<0.05$ CB_1 WT and HET versus CB_1 KO controls, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ WT versus CB_1 HET mice, n=7-10 mice per genotype per drug.

Figure 13 shows hypothermic effects of A-834,735D (Figure 13A), WIN55,212-2 (Figure 13B), CP55,940 (Figure 13C), JWH-073 (Figure 13D), CP47,497 (Figure 13E), and THC (Figure 13F). Each cannabinoid, with the exception of THC, produced dose-dependent hypothermia in WT and HET mice, but not in KO mice. THC produced significant hypothermia in all three genotypes, but effects in HET and KO mice were similar to each other and less in magnitude than those in WT mice. Table 3 shows ED_{50} values and dose ratios for all compounds. All drugs were more potent in WT mice than in HET mice. Neither the ED_{50} value nor the dose ratios (WT/HET mice) could be could be calculated for THC in HET mice because the magnitude of hypothermia did not achieve the level required (-4°C) required for calculation.

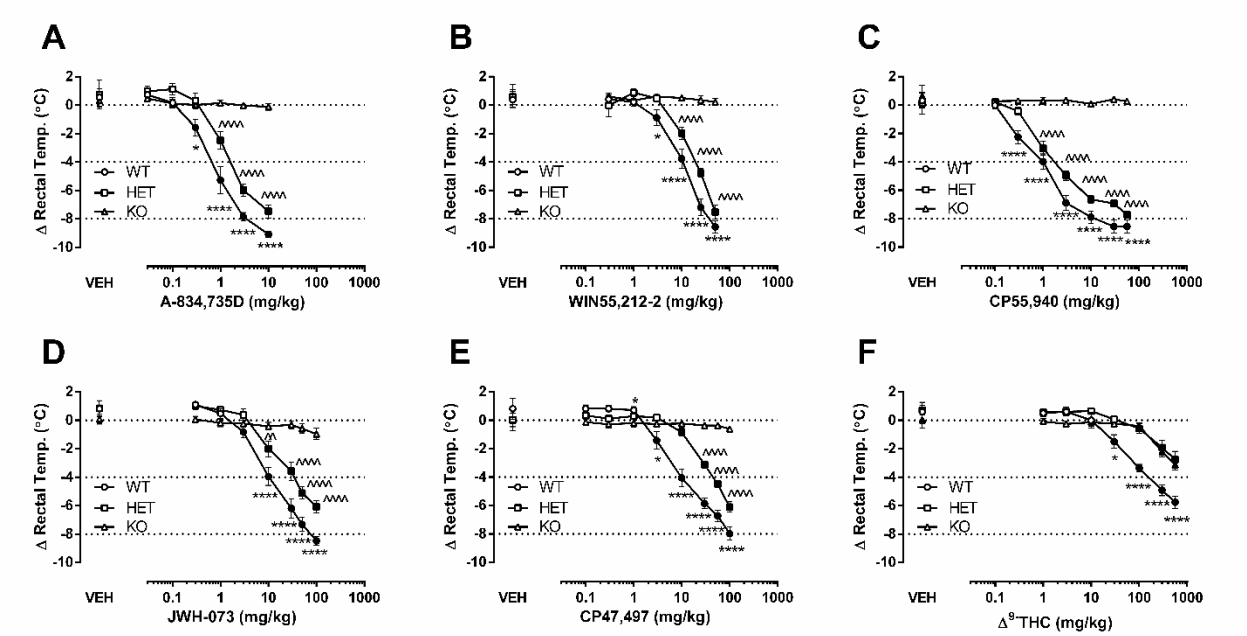

Figure 13. Differential potencies of THC and SCs between CB_1 WT and HET mice in producing hypothermia. A-834,735D (**A**, PR (95% CL) = 1.82 (1.44-2.34)), WIN55,212-2 (**B**, PR (95% CL) = 1.80 (1.34-2.45)), CP55,940 (C, PR (95% CL) = 2.05 (1.54-2.76)), and JWH-073 (**D**, PR (95% CL) = 2.74 (2.00-3.81)) produced similar, significant shifts in potency, while CP47,497 (**E**, PR (95% CL) = 3.55 (2.69-4.67)) produced an increased rightward shift relative to A-834,735D and WIN55,212-2. THC-induced hypothermia (**F**) showed an apparent loss of efficacy in CB_1 HET mice, with CB_1 HET and KO mice showing identical drops in body temperature (2.9 ± 0.5 C and 2.8 ± 0.4 C, respectively) at THC (560 mg/kg). The pre-injection mean ± SEM rectal temperatures for all groups was 36.72 ± 0.05 C. Data are expressed as a change from baseline. VEH indicates an injection of 1:1:18 vehicle prior to cumulative dosing with the indicated drug. Filled shapes indicate $p<0.05$ CB_1 WT and HET versus CB_1 KO controls, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ WT versus CB_1 HET mice, n=7-10 mice per genotype per drug.

Figure 15 shows cataleptic effects of each drug in WT, HET and KO mice. Each drug produced dose-dependent catalepsy in WT and HET mice, but did not produce significant effects in KO mice. Table 3 shows that ED_{50} values did not differ between WT and HET mice for any drug, and dose ratios (WT ED_{50}/HET ED_{50}) ranged from 0.56 to 1.15.

Figure 16 depicts the *in vivo* pharmacological effects of morphine and chlorpromazine in each genotype. Morphine produced dose-dependent hypothermia and antinociception that was equivalent in WT, HET, and KO mice. Chlorpromazine produced dose-related catalepsy and hypothermia irrespective of genotype. Figure 16 also shows that repeated vehicle injections generally was without effect in each genotype, with the exception of KO mice, which displayed a small increase in catalepsy after the fifth vehicle injection. Figures 17-23 show the time courses of single doses of each agonist and vehicle on each endpoint in WT and HET mice. All drugs had onsets of action by 30 min and durations of action ranging from 3-6 h. Table 5 contains results from ANOVAs for each drug and cannabinoid triad endpoint.

3.2 Radioligand binding and agonist-stimulated [^{35}S]GTPγS binding in CB_1 WT, HET, and KO mice

[^{3}H]SR141716A binding experiments (Table 6) confirmed high and low CB_1 expression in cerebellum and spinal cord, respectively, with CB_1 HET possessing approximately half of the number of receptors compared with WT mice in each case. Cerebellum and spinal cord membranes from CB_1 WT, HET, and KO mice were assessed for differences in maximal stimulation of GTPγS binding. Consistent with previous results (Breivogel *et al*, 2001; Monory *et al*, 2002), WIN55,212-2 elicited significant stimulation in CB_1 KO samples in cerebellum

(Figure 24, Table 7); therefore, CB_1 KO stimulation was subtracted to eliminate non-CB_1 mediated stimulation from WT and HET binding dose-effect curves (Figure 25). Nonlinear regression analyses of the curves prior to subtraction are shown in Table 7. None of the other ligands stimulated G protein activity in cerebellar homogenates from CB_1 KO mice. In cerebellum membranes (Table 4), A-834,735D ($T(6) = 2.544$, $p<0.05$), WIN55,212-2 ($T(6) = 5.482$, $p<0.01$), CP55,940 ($T(6) = 3.474$, $p<0.05$), JWH-073 ($T(6) = 20.97$, $p<0.0001$), CP47,497 ($T(6) = 6.837$, $p<0.001$) and THC ($T(6) = 4.442$, $p<0.01$) had significantly lower E_{max} values in CB_1 HET membranes than in WT membranes. THC yielded a significantly reduced E_{max} relative to the other ligands ($F(5,18) = 23.14$, $p<0.0001$). In spinal cord (A-834,735D ($T(6) = 5.826$, $p<0.01$), WIN55,212-2 ($T(6) = 4.283$, $p<0.01$), CP55,940 ($T(6) = 2.601$, $p<0.05$), and CP47,497 ($T(6) = 5.332$, $p<0.01$) E_{max} values were significantly lower in membranes from CB_1 HET than in WT membranes (see Table 2). Statistical differences were not detected between CB_1 HET and WT spinal membranes for JWH-073 ($p=0.052$) or THC ($p = 0.11$), the latter of which failed to produce stimulation above basal levels in CB_1 HET mice. Across agonists in WT samples, THC ($F(5, 18) = 15.26$, $p<0.0001$) produced significantly less stimulation than each other ligand, while none of the other drugs differed from one another.

3.3 Correlation of *in vivo* and *in vitro* measures of agonist efficacy

Figure 14 shows correlations between *in vivo* dose ratios (WT ED_{50}/HET ED_{50}) from each behavioral assay and *in vitro* WT E_{max} from [^{35}S]GTPγS binding assays in cerebellum and spinal cord membranes. Pearson correlation coefficients are shown in Table 4. HET and WT binding showed strong correlations between cerebellum and spinal cord. Catalepsy, hypothermia, and antinociception did not correlate with cerebellum binding. However, both hypothermia and antinociception highly correlated with [^{35}S]GTPγS binding in spinal cord.

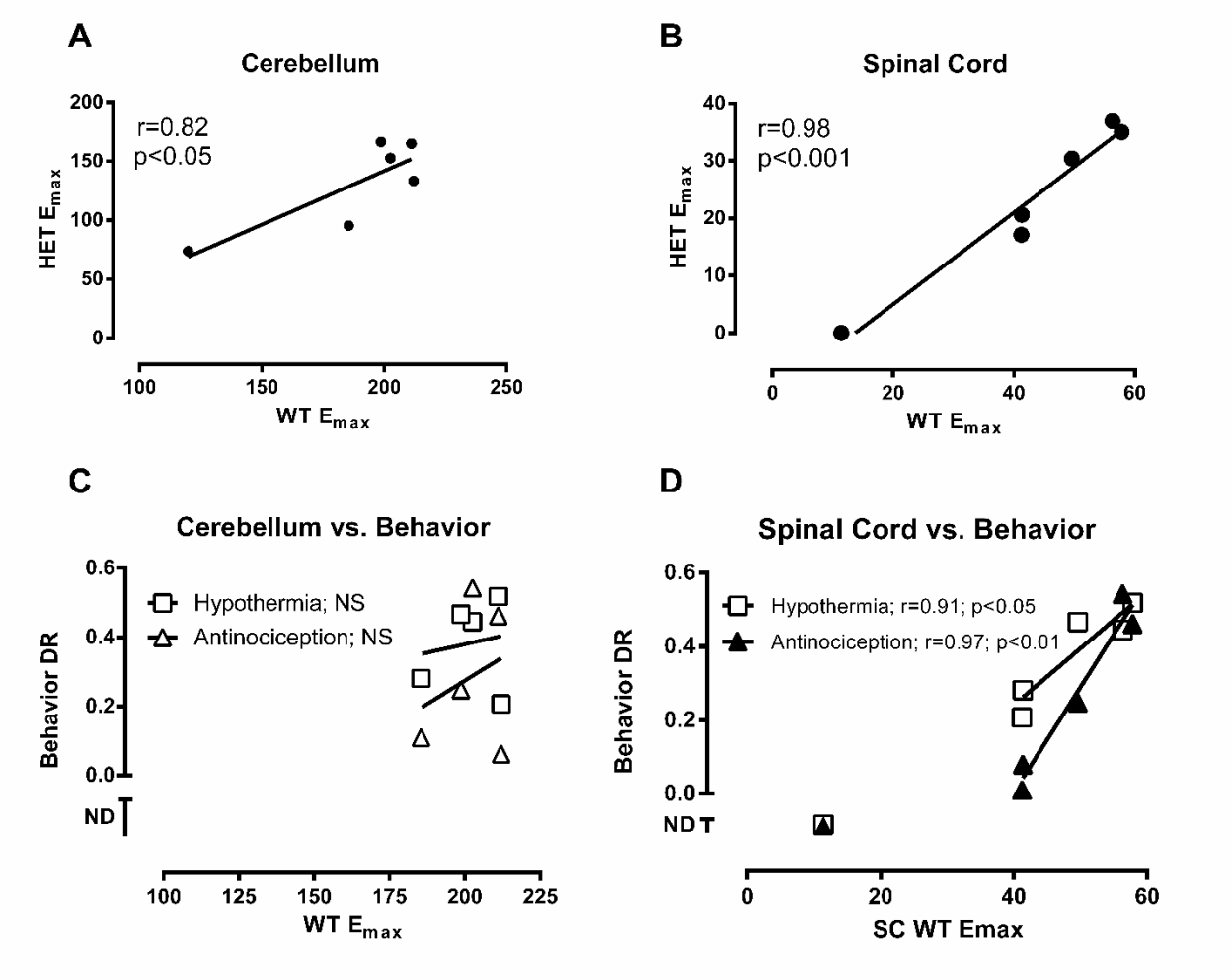

Figure 14. Correlations between in vivo potency differences between WT and HET mice and *in vitro* E_{max} values from agonist-stimulated [^{35}S]GTPγS binding experiments were assessed to elucidate whether triad measures reflected differences in functional CB_1 activity. In both cerebellum (**A**) and spinal cord (**B**), WT Emax correlated with HET Emax demonstrating the reduction in receptor population results in a concordant reduction in the Emax magnitude. Cerebellum WT Emax values were not correlated with catalepsy, hypothermia, or antinociception (**C**) while spinal cord WT Emax values were highly correlated with in vivo losses of potency between WT and HET mice for both hypothermia (r=0.91; p<0.05) and antinociception (r=0.97; p<0.01) (**D**).

4 Discussion

The cumulative dose-response cannabimimetic screen utilizing CB_1 transgenic mice that express varying levels of CB_1 receptors provides a high throughput *in vivo* method to discern relative differences in agonist selectivity and efficacy. The results presented here extend knowledge regarding the application of receptor theory to modern pharmacological challenges regarding cannabinoids, as it applies to the CB_1 receptor. Specifically, we elucidated the impact of reducing total CB_1 population on the relationship between *in vivo* potency/efficacy and *in vitro* functional activity.

Of the three *in vivo* pharmacological effects measured, antinociception was the most sensitive to reduction in receptor density. Specifically, CB_1 HET mice showed the greatest reductions in antinociceptive potency and efficacy to the cannabinoids tested compared with WT mice. A-834,735D, WIN55,212-2, and CP55,940 produced full antinociceptive effects in WT and HET mice, but CB_1 HET mice displayed 2 fold decreases in potency to A-834,735D, WIN55,212-2, and ~7 fold decrease in potency to CP55,940. THC was the least potent cannabinoid in producing antinociception in WT mice, and failed to achieve greater than 25% MPE in CB_1 HET mice, rendering the ED_{50} value incalculable. Similarly, the dose-response analyses of JWH-073 and CP47,497 within the dose ranges tested revealed reduced magnitudes of antinociceptive effects in HET mice compared with WT mice. KO mice did not display relevant antinociception following administration of any of the cannabinoids. In contrast, the non-cannabinoid morphine produced full antinociceptive effects irrespective of genotype, while chlorpromazine did not produce antinociception. CB_1 density is relatively low in CNS areas purported to mediate antinociception (e.g., periaqueductal gray, dorsal horn of the spinal cord) compared to other brain regions, such as the cerebellum (Herkenham *et al*, 1990, 1991; Matsuda

et al, 1990). Thus, these findings indicate relatively low CB_1 receptor reserve for antinociception. Accordingly, THC, CP47,497, and JWH-073 behaved as low efficacy CB_1 agonists, while CP55,940 had higher efficacy, and A-834,735D and WIN55,212-2 were the highest efficacy compounds in producing antinociception.

Similar to antinociception, the dose response curve for each agonist in producing hypothermia was rightward shifted in CB_1 HET mice compared with WT mice. Interestingly, THC-induced hypothermia showed a profound reduction of E_{max} in CB_1 HET mice, with body temperature only partially reduced at 300 and 560 mg/kg THC. The observation that body temperature drops did not differ between HET and KO mice suggests off-target effects at these excessively high concentrations of THC. However, none of the other cannabinoids, at the doses assessed, produced hypothermia in CB_1 KO mice. Morphine and chlorpromazine elicited dose-dependent hypothermia irrespective of genotype (Figure 16). These findings taken together with the reduced level of CB_1 expression in the POA (Herkenham *et al*, 1990) suggest relatively low CB_1 receptor reserve for cannabinoid-induced hypothermia.

In contrast to the antinociceptive and hypothermic measures, WT and HET mice displayed similar dose-response relationships to the cataleptic effects of the six cannabinoid tested (Figure 15), suggesting a relatively high CB_1 receptor reserve. Chlorpromazine produced catalepsy in all three genotypes, while morphine did not elicit catalepsy (Figure 16). The minimal rightward shift in the dose-response relationship of cannabinoids in CB_1 HET mice is consistent with idea that the high levels of CB_1 expression in brain areas mediating this behavior yield sufficient receptor reserve. Indeed, dorsal striatum (~3-4 pmol/mg) and cerebellum (4-6 pmol/mg) CB_1 expression represent among the highest levels in brain (Selley *et al*, 2001). Work from Dhawan et al. 2006 suggests cannabinoid-induced catalepsy requires low CB_1 occupancy.

Accordingly, a 50% reduction in receptor expression (Table 6) is likely insufficient to decrease ligand potency and efficacy in producing catalepsy. Similarly, THC, a low efficacy CB_1 agonist as determined in agonist-stimulated [^{35}S]GTPγS binding experiments, elicited catalepsy that differed little between CB_1 HET and WT mice. Interestingly, small, but significant differences between WT and HET mice were found for A-834,735D, WIN55,212-2, CP55,940, and THC, but not for JWH-073 and CP47,497. This pattern of effects does not follow the expected efficacy continuum, but rather may be mediated by other aspects of the ligand, such as alternative and/or additional signaling mechanisms, in addition to the canonical $G\alpha_{i/o}$ and downstream cAMP inhibition pathway.

In the present study, we determined dose-response relationships of each drug using a cumulative dosing within subject procedure, which allowed a fairly high throughout with a reduced total number of mice required (i.e., the entire dose-range was tested in each mouse during a single session). Consequently, the possibility of tachyphylaxis occurring during the cumulative dosing procedure may have contributed to potency reductions in HET mice, especially for measures that may be mediated by low CB_1 receptor reserve. However, the time-course studies (Figures 17-23) suggest a relatively long duration of action for each cannabinoid tested in both WT and HET. Thus, even if the dosing regimen leads to adaptive changes at CB_1 within the timeframe of the assay, the conditions were relatively similar across drugs and genotypes. Although the assessment of pharmacokinetic factors was beyond the scope of the present study, we previously reported similar blood and brain THC levels resulting from single bolus injection and cumulative dosing regimen at 10, 30, and 56 mg/kg (Falenski *et al*, 2010). Nonetheless, as CB_1 agonists vary profoundly in structure, with endogenous cannabinoids

undergoing rapid hydrolysis within seconds or minutes (Blankman and Cravatt, 2013), future studies may need to take pharmacokinetic factors into consideration.

Agonist-stimulated [^{35}S]GTPγS binding experiments generally corroborated the *a priori* selection of CB_1 agonists, which vary from high to low efficacy (A-834,735D ≥ WIN55,212-2 > CP55,940 > JWH-073 ≥ CP47,497 > THC), when relative E_{max} differences of WT and HET mice were taken into account. This continuum was consistent under high (i.e., cerebellum homogenates) and low CB_1 (i.e., spinal cord homogenates) expression conditions. The absence of a correlation between catalepsy and GDP-GTP exchange is consistent with the idea of high CB_1 receptor reserve. Significant correlations were detected between *in vitro* WT Emax values from [^{35}S]GTPγS binding experiments in spinal cord and the *in vivo* hypothermia and antinociception measures, suggest that low receptor conditions reveal stratification of ligands by efficacy.

One issue these assays do not address is potential signaling events outside of the canonical G-protein-cAMP pathway. Of the ligands tested here, potential bias has been described for CP55,940, WIN55,212-2, and THC in striatal cell cultures (Khajehali *et al*, 2015; Laprairie *et al*, 2014), though more work remains to be done in this emerging area. CB_1 ligands with extreme bias for one pathway or another would be highly useful to test whether alternative signaling pathways play determining roles in the *in vivo* potency and efficacy of cannabinoids. For instance, the endogenous cannabinoid/endogenous TRPV1 agonist N-arachidonoyl dopamine (Redmond *et al*, 2015) preferentially modulates Ca^{2+} via $G\alpha_q$, though this compound may not be a good candidate for *in vivo* testing due to its likely rapid hydrolysis. Future studies may focus on investigating where novel, abused SCs fall along the efficacy continuum. Utilizing ligands with extreme bias may be particularly revealing in this assay. Although not commercially available,

irreversible CB_1 antagonists would also provide great utility to investigate CB_1 receptor density across relevant endpoints. This approach has already been implemented successfully for the mu opioid receptor (Madia *et al*, 2009; Pawar *et al*, 2007; Walker *et al*, 1998).

In conclusion, the present study establishes a high throughput, within subjects approach to assess *in vivo* efficacy of SCs as well as naturally occurring cannabinoids by assessing their pharmacological effects in established assays using CB_1 WT, HET, and KO mice. In particular, the strong relationship between loss of efficacy of cannabinoids in GTPγS binding in spinal cord tissue and potency reductions of the six cannabimimetic ligands in producing antinociception and hypothermia in CB_1 HET mice suggests that these endpoints reflect good predictors of *in vivo* efficacy. The lack of correlation between GTPγS binding and catalepsy is likely due to the high number of spare CB_1 receptors in brain regions mediating this pharmacological effect, which is consistent with the small reduction in potency observed in HET mice. The present study describes a solid preclinical approach, based on pharmacological principles, to provide valuable insight into the pharmacology of emerging abused SCs. More generally, the use of CB_1 transgenic mice through examination of dose-response relationships of cannabinoids on antinociception and hypothermia as well as on agonist-stimulated GTPγS binding in spinal cord tissue possesses utility in determining *in vivo* and *in vitro* efficacy of emerging abused SCs, cannabinoids being investigated for basic research or being developed as potential medications, and naturally occurring cannabinoids.

Antinociception	WT ED_{50} (95% CL)	HET ED_{50} (95% CL)	Dose Ratio (WT/HET)
A-834,735D	0.85 (0.61-1.19)	1.84 (1.66-2.04)*	0.46
WIN55,212-2	9.64 (6.44-14.41)	17.77 (10.61-29.77)	0.54
CP55,940	1.00 (0.66-1.51)	4.04 (2.69-6.09)*	0.24
JWH-073	7.74 (6.08-9.84)	96.58 (47.41-196.76)*	0.08
CP47,497	6.20 (4.22-9.10)	100 mg/kg: 44.3±11.2 %MPE*	<0.06
THC	81.69 (51.32-130.03)	560 mg/kg: 19.2±2.7 %MPE*	<0.15
Hypothermia	**WT ED_{50} (95% CL)**	**HET ED_{50} (95% CL)**	**Dose Ratio (WT/HET)**
A-834,735D	0.85 (0.67-1.00)	1.64 (1.59-1.69)*	0.52
WIN55,212-2	6.97 (5.24-9.29)	15.68 (9.95-24.71)*	0.45
CP55,940	1.06 (0.74-1.51)	2.27 (1.85-2.79)*	0.47
JWH-073	8.75 (6.99-10.94)	31.16 (21.08-46.06)*	0.28
CP47,497	9.84 (7.81-12.39)	47.53 (32.29-69.97)*	0.21
THC	155.67 (120.63-200.88)	560 mg/kg: -2.75±0.57ºC*	<0.28
Catalepsy	**WT ED_{50} (95% CL)**	**HET ED_{50} (95% CL)**	**Dose Ratio (WT/HET)**
A-834,735D	0.82 (0.62-1.08)	0.94 (0.73-1.21)	0.87
WIN55,212-2	4.06 (3.09-5.34)	5.75 (4.43-7.47)	0.71
CP55,940	0.93 (0.18-1.83)	0.81 (0.50-1.29)	1.15
JWH-073	7.72 (5.97-9.99)	8.03 (6.01-10.73)	0.96
CP47,497	3.68 (1.81-7.51)	6.57 (4.15-10.38)	0.56
THC	28.36 (17.99-44.72)	49.27 (36.35-66.78)	0.58

Table 3. The antinociceptive and hypothermic effects of each ligand were more potent in WT mice than in HET mice, with the exception of WIN55,212-2-induced antinociception, which did not significantly differ between genotypes. For catalepsy, none of the ligands differed in potency between WT and HET mice. ED_{50} values (expressed in mg/kg) for each dependent measure were calculated in WT and HET mice. Dose ratios were calculated by dividing the WT ED_{50} by the HET ED_{50}. In the cases in which ED_{50} estimations were not possible to calculate (i.e., CP47,497 for antinociception, and THC for antinociception and hypothermia in HET mice), the maximum

effect at the highest dose tested is presented. * indicates non-overlapping confidence intervals between WT and HET ED_{50} values.

Cerebellum

	WT		HET		
Agonist	E_{max}	EC_{50}	E_{max}	EC_{50}	HET E_{max}/ WT E_{max}
A-834,735D	211.1 ± 11.4	22.3 ± 5.6	164.9 ± 14.1*	29.4 ± 11.9	0.78
WIN55,212-2	202.5 ± 7.6	39.4 ± 7.6	152.6 ± 4.9**	50.4 ± 8.0	0.75
CP55,940	198.7 ± 5.2	4.1 ± 0.6	166.3 ± 7.6*	6.1 ± 1.4	0.84
JWH-073	185.5 ± 1.7	25.6 ± 1.2	95.5 ± 3.9****	38.3 ± 7.1	0.51
CP47,497	212 ± 8.6	102.1 ± 18.2	133.3 ± 7.6***	94.5 ± 22.8	0.63
THC	119.9 ± 4.6^	24.3 ± 4.7	73.8 ± 8.7**	52.4 ± 29.0	0.62

Spinal Cord

	WT		HET		
Agonist	E_{max}	EC_{50}	E_{max}	EC_{50}	HET E_{max}/ WT E_{max}
A-834,735D	57.8 ± 2.4	15.0 ± 3.0	35.0 ± 3.0**	24.1 ± 9.6	0.61
WIN55,212-2	56.3 ± 3.8	65.4 ± 22.0	36.9 ± 2.4**	84.8 ± 21.6	0.66
CP55,940	49.6 ± 5.9	8.61 ± 5.1	30.4 ± 4.3*	9.5 ± 6.9	0.61
JWH-073	41.3 ± 3.5	77.8 ± 29.9	20.6 ± 7.7	46.6 ± 91.0	0.49
CP47,497	41.2 ± 3.9	93.2 ± 38.1	17.1 ± 2.2**	45.9 ± 24.2	0.42
THC	11.4 ± 2.3^	6.1 ± 11.2	ND	ND	ND

Correlations

Cerebellum	r	p
Catalepsy	0.10	0.53
Hypothermia	0.02	0.80
Antinociception	0.08	0.65

Spinal Cord	r	p
Catalepsy	0.22	0.34
Hypothermia	0.84	<0.05
Antinociception	0.95	<0.01

Table 4. Each agonist was assessed for its ability to stimulate [^{35}S]GTPγS binding in cerebellum and spinal cord membranes of CB_1 WT, HET, and KO mice. In cerebellum, all agonist elicited comparatively lower stimulation in HET membranes than in WT membranes. Comparison of WT E_{max} values across drugs revealed that THC elicited the least stimulation of all ligands tested. Spinal cord membranes revealed a similar pattern of results, with only JWH-073 failing to elicit significant differences in E_{max} between WT and HET samples. No stimulation above basal

was detected for THC in HET spinal cord; therefore, E_{max} and EC50 values could not be determined (ND). Similar to cerebellum, only THC displayed significantly lower E_{max} values across WT samples in spinal cord. This loss of efficacy is expressed additionally as the HET E_{max}/WT E_{max} to show relative differences between the higher receptor conditions of cerebellum and low receptor conditions of spinal cord. ^indicates significant differences versus all other ligands within WT E_{max} values, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 for WT versus HET samples within tissue. No significant differences in EC50 were detected across brain region or genotype for each drug.

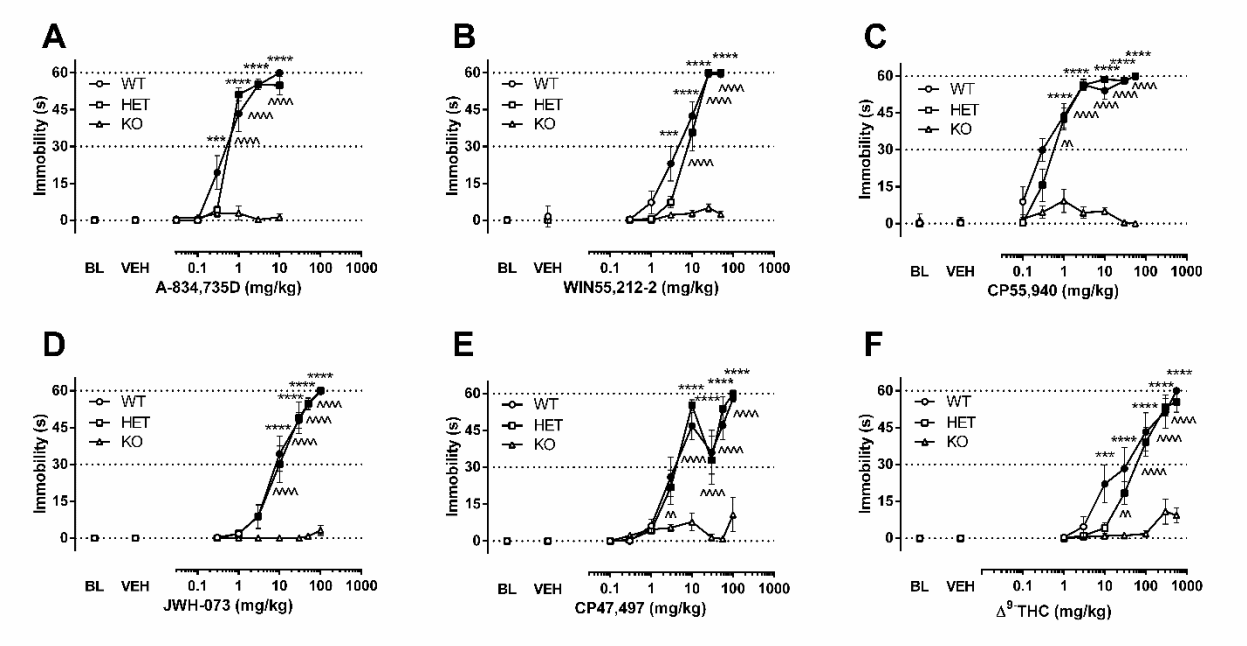

Figure 15. All drugs tested maintained comparable potency in the catalepsy measure of the triad in HET mice compared to their WT counterparts. There were significant dose by genotype interactions for A-834,735D (0.03-10 mg/kg, Figure 1A, $F(14, 147) = 41.99$, $p<0.0001$), WIN55,212-2 (0.3-50 mg/kg, Figure 1B, $F(14, 133) = 31.69$, $p<0.0001$), CP55,940 (0.03-56 mg/kg, Figure 1C, $F(16,168) = 29.84$, $p<0.0001$), JWH-073 (0.3-100 mg/kg, Figure 1D, $F(16, 160) = 22.91$, $p<0.0001$), CP47,497 (0.1-100 mg/kg, Figure 1E, $F(18, 189) = 10.75$, $p<0.0001$), and THC (1-560 mg/kg, Figure 1F, $F(16, 176) = 13.70$, $p<0.0001$). Table 1 shows no significant differences between WT and HET mice across drugs. BL indicates baseline and VEH indicates an injection of 1:1:18 vehicle prior to cumulative dosing with the indicated drug. Filled shapes indicate $p<0.05$ versus respective vehicle for each genotype, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ WT versus KO, ^$p<0.05$, ^^$p<0.01$, ^^^$p<0.001$, ^^^^$p<0.0001$ HET versus KO, n=7-10 mice per genotype per drug.

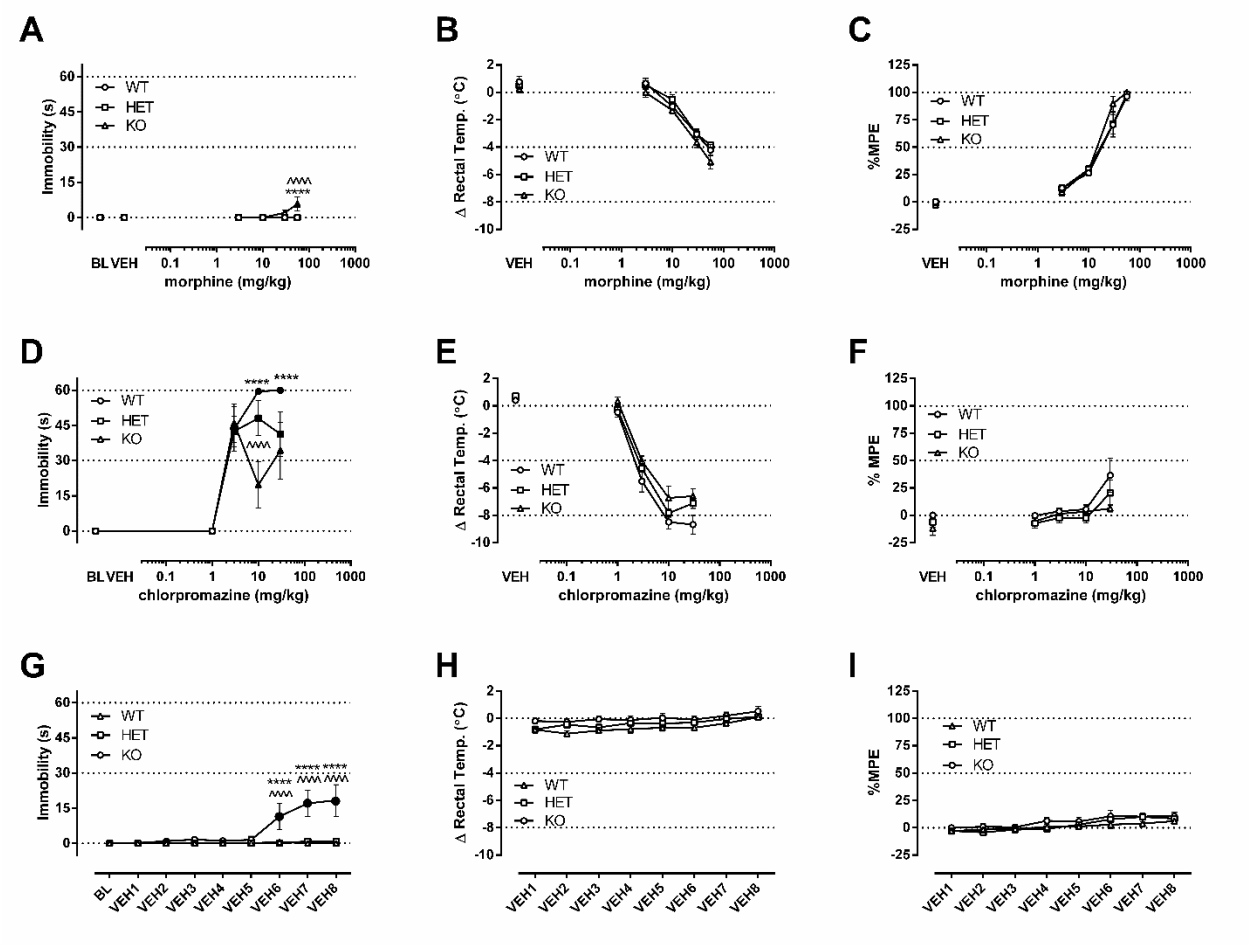

Figure 16. The mu opioid receptor agonists morphine elicits catalepsy in CB_1 KO, but not WT or HET mice, at a cumulative dose of 56 mg/kg (**A**, F (10, 95) = 4.545, p<0.0001). Morphine also, produced dose-dependent hypothermia (**B**, main effect of morphine p<0.0001, no significant interaction p=0.7285) and antinociception (**C**, main effect of morphine p<0.0001, no significant interaction p=0.2545) irrespective of genotype. Cumulative dosing with the antipsychotic chlorpromazine resulted in dose-dependent increases in catalepsy (**D**, main effect of chlorpromazine, p<0.0001, no significant interaction p=0.9239) measured as well as dose-dependent decreases in body temperature (**E**, main effect of chlorpromazine p<0.0001, no significant interaction p=0.5727) with no significant increases in antinociception (**F**, main effect

of chlorpromazine, $p<0.0001$, no significant interaction $p=0.4886$). To control for repeated injections, six single volume injections of vehicle followed by two double volume injections were administered. Significant increases in catalepsy were measured in CB1 KO mice (**G**, $F(16, 168) = 6.396$, $p<0.0001$) though it is unclear whether this is due to repeated injections or habituation to the bar test itself. No significant changes were detected in hypothermia (**H**, $p=0.4708$) nor antinociception (**I**, $p=0.4935$). ****$p<0.0001$ versus WT and HET, n = 7-9. Filled shapes indicate $p<0.05$ versus respective vehicle for each genotype, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ WT versus KO, ^$p<0.05$, ^^$p<0.01$, ^^^$p<0.001$, ^^^^$p<0.0001$ HET versus KO, n=7-10 mice per genotype per drug.

Antinociception	F ratio	p value
A-834,735D	F (12, 126) = 15.33	<0.0001
WIN55,212-2	F (12, 114) = 15.79	<0.0001
CP55,940	F (14, 147) = 21.52	<0.0001
JWH-073	F (14, 140) = 20.98	<0.0001
CP47,497	F (16, 168) = 10.98	<0.0001
THC	F (14, 154) = 18.74	<0.0001
Hypothermia	**F value**	**p value**
A-834,735D	F (12,126) = 67.78	<0.0001
WIN55,212-2	F (12,114) = 37.42	<0.0001
CP55,940	F (14, 147) = 77.85	<0.0001
JWH-073	F (14,140) = 41.82	<0.0001
CP47,497	F (16, 168) = 72.02	<0.0001
THC	F (14,154) = 13.84	<0.0001
Catalepsy	**F value**	**p value**
A-834,735D	F (14, 147) = 41.99	<0.0001
WIN55,212-2	F (14, 133) = 31.69	<0.0001
CP55,940	F (16,168) = 29.84	<0.0001
JWH-073	F (16, 160) = 22.91	<0.0001
CP47,497	F (18, 189) = 10.75	<0.0001
THC	F (16, 176) = 13.70	<0.0001

Table 5. The results from the ANOVAs of each cannabinoid for each dependent measure of the cumulative dosing triad are depicted here. The results of post hoc Holms-Sidak analysis are depicted in the Figure 12, Figure 13, and Figure 15.

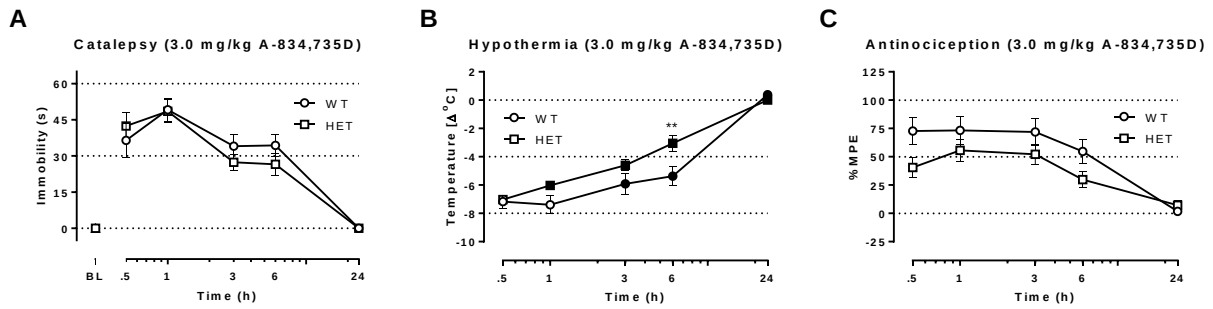

Figure 17. A-834,375D (3.0 mg/kg) produced cataleptic (**A**, F (5, 110) = 0.8960, p = 0.4866, no significant interaction; main effect of time, p<0.0001), hypothermic (**B**, F (4, 88) = 4.765, p < 0.01), and antinociceptive (**C**, F (4, 88) = 1.521, p = 0.2028, no significant interaction; main effect of time, p < 0.0001) effects in a time dependent manner, revealing a long duration of action peaking at one hour post-injection in all three measures. CB_1 WT mice only differed from HET mice at six hours post-injection in hypothermia. **p<0.01, n = 12.

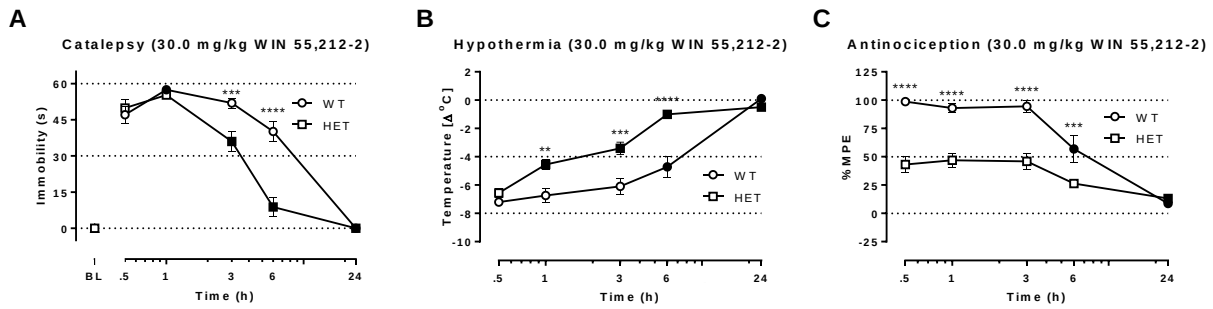

Figure 18. A dose (30.0 mg/kg) of CB_1 agonist WIN 55,212-2 produced cannabimimetic effects in a time-dependent manner. Separation between genotypes (indicated by asterisks) is readily apparent after three hours in catalepsy (**A**, $F(5, 100) = 13.74$, $p < 0.0001$) and after one hour in hypothermia (**B**, $F(4, 80) = 12.46$, $p < 0.0001$) and antinociception (**C**, $F(4, 80) = 9.929$, $p < 0.0001$). HET mice approached baseline measurements in catalepsy and antinociception six hours post-injection while WT mice remained near or above peak measurements three hours post-injection in all three measures before declining at six hours post-injection. Between genotypes, significant differences were found at half an hour post-injection in antinociception, one hour in hypothermia and antinociception), three hours in all three measures, and six hours in all three measures. **p<0.01, ***p<0.001, ****p < 0.0001, n = 8-10.

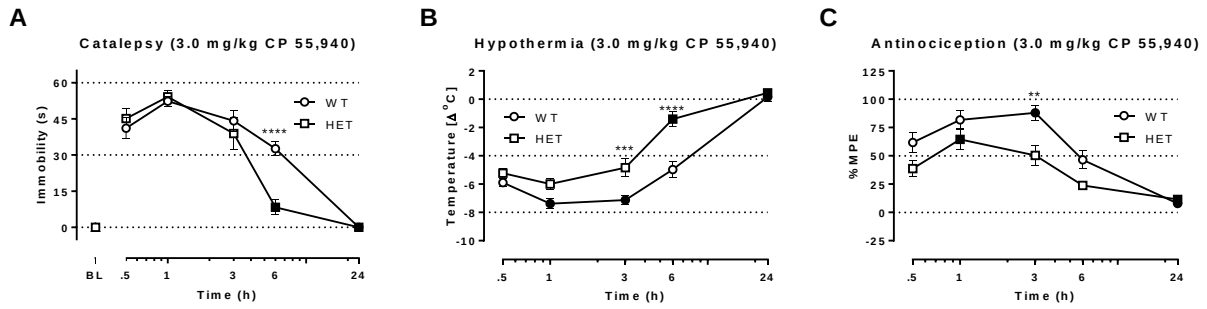

Figure 19. Cannabinoid triad time course of CP 55,940 (3.0 mg/kg) showed peak cannabimimetic activity in both CB_1 WT and HET mice at one hour post-injection in catalepsy (**A**, $F(5, 110) = 5.875$, $p < 0.0001$) and hypothermia (**B**, $F(4, 88) = 8.668$, $p < 0.0001$) and three hours post-injection in antinociception (**C**, $F(4, 88) = 2.945$, $p < 0.05$), indicating time dependent effects. Hypothermia and antinociception displayed significant differences between genotypes at three hours post injection. Catalepsy and hypothermia showed significant differences between genotypes at six hours post-injection. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, $n = 12$.

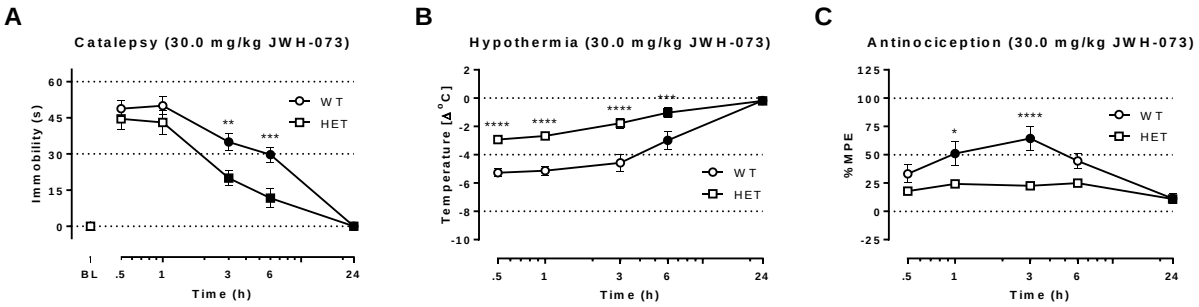

Figure 20. JWH-073 (30.0 mg/kg) elicited cataleptic (**A**, $F(5, 90) = 3.025$, $p < 0.05$), hypothermic (**B**, $F(4, 72) = 5.830$, $p<0.001$), and antinociceptive (**C**, $F(4, 72) = 5.239$, $p < 0.001$) effects in a time dependent manner. Significant differences between CB_1 WT and HET mice are discernible at half an hour post-injection in hypothermia, one hour post-injection in antinociception, and three hours post-injection in catalepsy. Differences are continuous to six hours post-injection in catalepsy and hypothermia, and three hours in antinociception. HET mice extinguished cannabimimetic behavior in all three measures earlier than WT mice. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, $n = 10$.

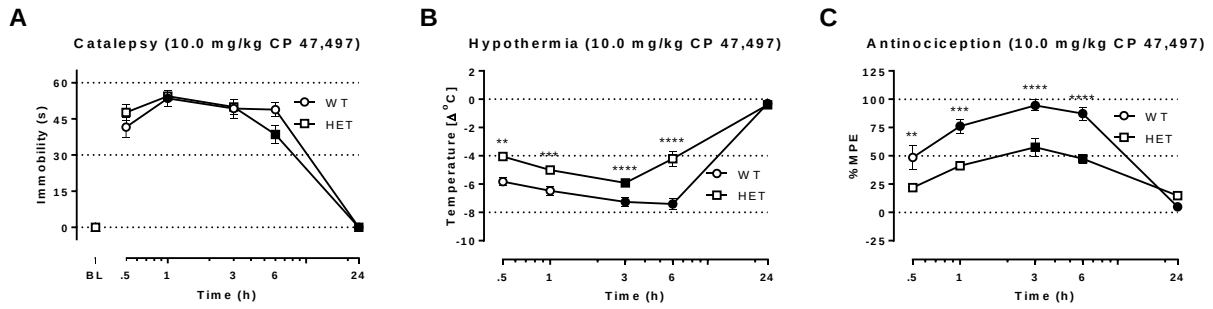

Figure 21. CP 47,497 displayed a long, consistent duration of action in CB_1 WT and HET mice in cannabinoid triad as evidenced by an absence of significant differences in measurements from the half hour time point (indicated by filled symbols) until six hours post-injection in catalepsy (**A,** $F (5, 110) = 2.354$, $p = 0.0452$), and three hours post-injection in hypothermia (**B**, $F (4, 88) = 9.108$, $p < 0.0001$) and antinociception (C, $F (4, 88) = 7.576$, $p < 0.0001$). Separation between genotypes was observed in hypothermia and antinociception beginning half an hour post-injection until six hours post-injection. **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, $n = 12$.

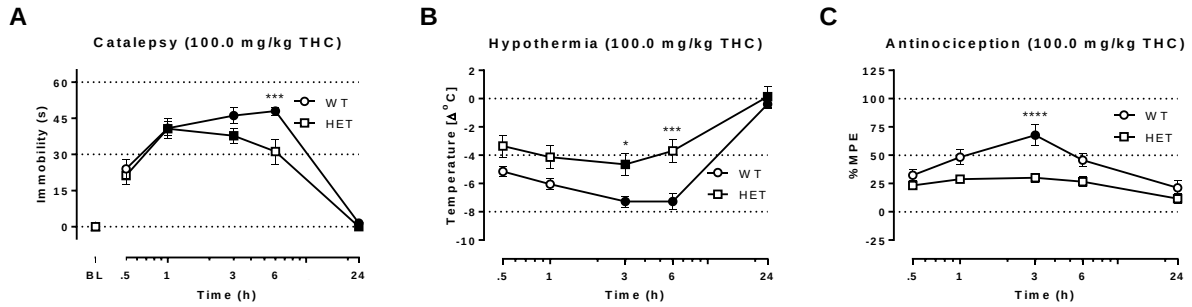

Figure 22. THC (100.0 mg/kg) achieved peak cannabimimetic activity time dependently in CB_1 WT mice in cannabinoid triad time course at six hours post-injection in catalepsy (**A**, F (5, 110) = 2.354, $p < 0.05$) and hypothermia (**B**, F (4, 88) = 9.108, $p < 0.0001$) and three hours post-injection in antinociception (**C**, F (4, 88) = 7.576, $p < 0.0001$) and in CB_1 HET mice at one hour post-injection in catalepsy and antinociception and three hours post-injection in hypothermia. WT mice differed from HET mice at three and six hours for hypothermia, at six hours in catalepsy, and at six hours for antinociception. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$, n = 11-13.

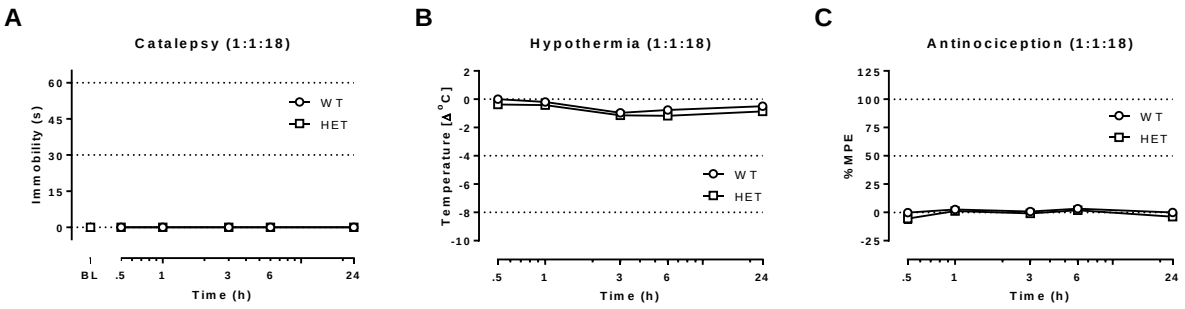

Figure 23. Vehicle (0.01 mL/g) produced no cannabimimetic effects in catalepsy (**A**), hypothermia (**B**, F (4, 88) = 0.437, p = 0.7812; no significant effect), or antinociception (**C**, F (4, 88) = 0.5342, p = 0.7109; no significant effect). n = 12.

	Cerebellum		Spinal Cord	
	B_{max} (pmol/mg)	K_D [nM]	B_{max} (pmol/mg)	K_D [nM]
CB_1 WT	4.32 ± 0.30	1.15 ± 0.14	0.97 ± 0.09	0.58 ± 0.12
CB_1 HET	1.89 ± 0.36***	0.95 ± 0.21	0.58 ± 0.10#	0.90 ± 0.34

Table 6. [^{3}H]SR141716A binding confirmed approximately 50% CB_1 expression in the brain areas tested in the agonist stimulated [^{35}S]GTPγS binding assay. The K_D of the radioligand did not change between genotypes or across CNS regions (p=0.3957). Binding curves were fitted with nonlinear regression in GraphPad Prism 6.0. ***p<0.001 WT versus HET B_{max} in cerebellum, #p<0.05 WT versus HET B_{max} in spinal cord, n = 8 for each genotype per tissue.

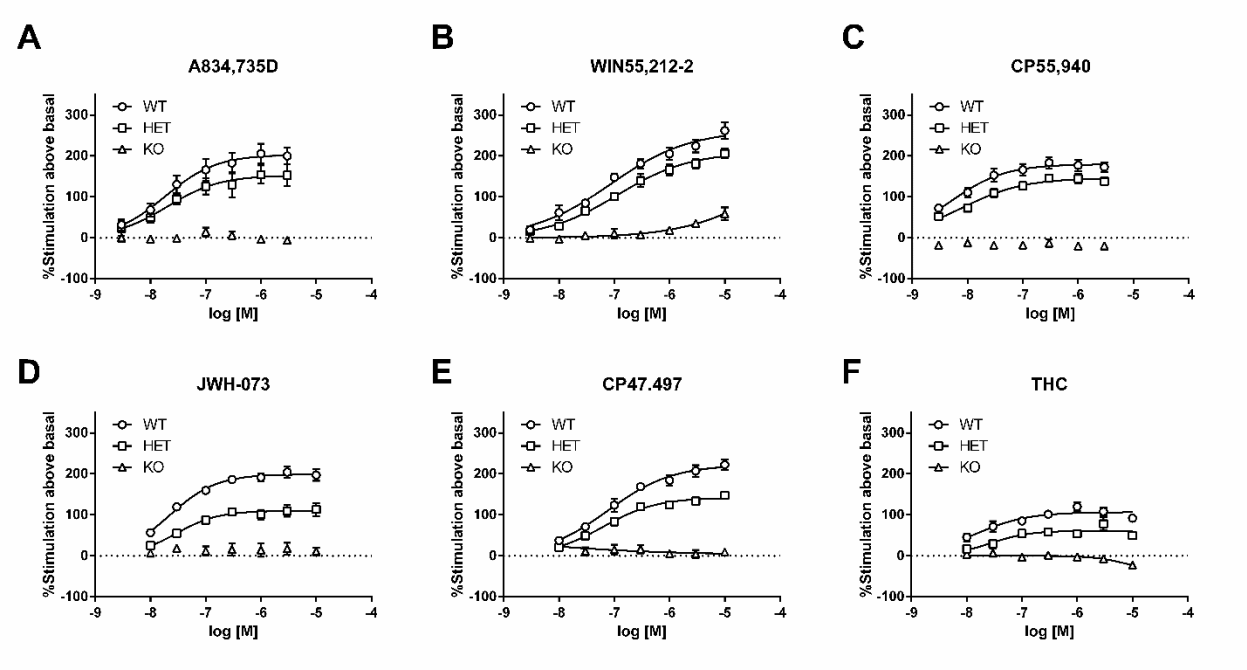

Figure 24. All agonists tested stimulated [^{35}S]GTPγS binding to a similar degree in wild type cerebellum, except for THC which displayed significantly lower E_{max} values in wild-type tissue (**Table 4**) and WIN55,212-2 which displayed much higher stimulation. A-834,735D (**A**), WIN55,212-2 (**B**), and CP55,940 (**C**) displayed similar differences in E_{max} among heterozygous tissue (~80% of wild type), while JWH-073(**D**), CP47,497 (**E**), and THC (**F**) each displayed close to a 50% in efficacy. Dose-related changes in agonist-stimulated binding were detected in knockout samples for both WIN55,212-2 ($F(7, 24) = 6.378$, $p<0.001$) and THC ($F(6, 21) = 6.492$), thus knockout stimulation was subtracted for the purposes of analysis from both wild type and heterozygous samples for each drug. EC_{50} and E_{max} values for the data here are shown in Table 7. ND indicates not determined, n = 4 per genotype.

	WT		HET		KO	
Agonist	E_{max}	EC_{50}	E_{max}	EC_{50}	E_{max}	EC_{50}
A-834,735D	203.0 ± 15.9	18.6 ± 6.7	152.7 ± 16.3	20.2 ± 9.9	ND	ND
WIN55,212-2	262.6 ± 18.6	86.4 ± 31.6	209.3 ± 13.5	114.5 ± 36.1	ND	ND
CP55,940	179.6 ± 8.1	5.0 ± 1.2	146 ± 6.8	7.8 ± 1.8	ND	ND
JWH-073	199.1 ± 5.9	22.5 ± 3.3	109.9 ± 6.2	29.7 ± 7.9	ND	ND
CP47,497	222.2 ± 11.3	78.0 ± 18.6	142.1 ± 4.3	63.3 ± 8.8	ND	ND
THC	106.6 ± 6.2	14.4 ± 4.6	61.21 ± 4.9	26.8 ± 10.1	ND	ND

Table 7. E_{max} and EC_{50} values calculated from agonist-stimulated GTPγS binding experiments in cerebellum which are uncorrected for KO stimulation. WIN55,212-2 achieved a maximum of 40% above basal in KO tissue whereas the other five ligands tested elicited no discernable stimulation above basal in KO tissue.

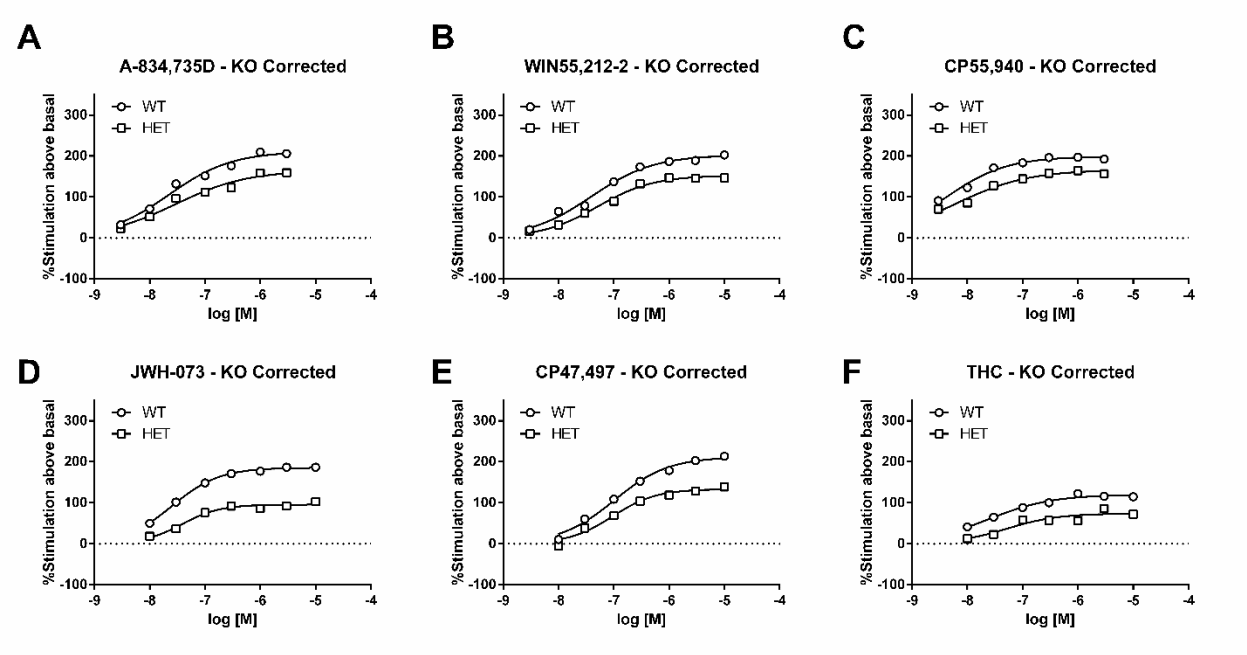

Figure 25. Subtraction of knockout stimulation revealed equal efficacy in WT tissue of all agonists except for THC. EC_{50} values in wild type and heterozygous tissue for WIN55,212-2 were much lower following subtraction, implying it may be more potent to activate CB_1 receptors than previous reported in cases where off target stimulation was not taken into account. EC_{50} and E_{max} values for the data here are shown in the table below. ND indicates not determined.

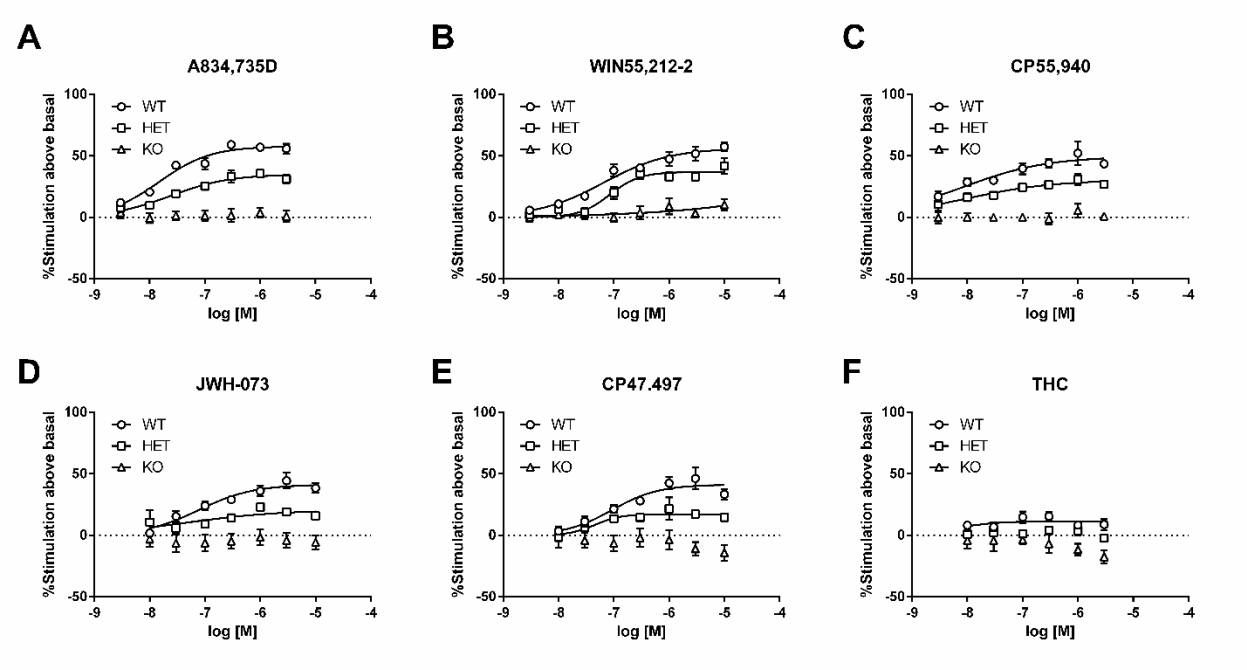

Figure 26. Unlike cerebellum, no significant stimulation was detected in knockout in spinal cord thus no subtraction was necessary. EC_{50} and E_{max} values for the data here are shown in the table below. ND indicates not determined, n = 4 per genotype.

Acknowledgements

The authors of this manuscript would like to thank Brittany Mason, Mohammed Mustafa, Pamela Weller, and Jolene Windle for their assistance with breeding the majority of the mice used for these studies. We would also like to thank the National Institute on Drug Abuse for their contribution of drugs used for these studies.

Grants

This research was supported by the following funding sources: T32DA007027, R01DA032933

Conflicts of Interest

The authors have no conflicts of interest to declare.

Chapter 3

Effects of acute and repeated dosing of the synthetic cannabinoid CP55,940 on intracranial self-stimulation in mice.

Title: Effects of acute and repeated dosing of the synthetic cannabinoid CP55,940 on intracranial self-stimulation in mice.

Article Type: Full Length Report

Corresponding author: Travis Grim

Corresponding Author's Institution: Virginia Commonwealth University

First Author: Travis Grim

Order of Authors: Travis W. Grim, Jason M. Wiebelhaus, Anthony Morales, S. Stevens Negus Aron H. Lichtman

As published in Drug and Alcohol Dependence: (Grim *et al*, 2015)

ABSTRACT

Background: Synthetic cannabinoids have emerged as a significant public health concern. To increase the knowledge of how these molecules interact on brain reward processes, we investigated the effects of CP55,940, a high efficacy synthetic CB_1 receptor agonist, in a frequency-rate intracranial self-stimulation (ICSS) procedure.

Methods: The impact of acute and repeated administration (seven days) of CP55,940 on operant responding for electrical brain stimulation of the medial forebrain bundle was investigated in C57BL/6J mice.

Results: CP55,940 attenuated ICSS in a dose-related fashion (ED50 (95% C.L.) = 0.15 (0.12-0.18) mg/kg). This effect was blocked by the CB_1 receptor antagonist rimonabant. Tolerance developed quickly, though not completely, to the rate-decreasing effects of CP55,940 (0.3 mg/kg). Abrupt discontinuation of drug did not alter baseline responding for up to seven days. Moreover, rimonabant (10 mg/kg) challenge did not alter ICSS responding in mice treated repeatedly with CP55,940.

Conclusions: The finding that CP55,940 reduced ICSS in mice with no evidence of facilitation at any dose is consistent with synthetic cannabinoid effects on ICSS in rats. CP55,940-induced ICSS depression was mediated through a CB_1 receptor mechanism. Additionally, tolerance and dependence following repeated CP55,940 administration were dissociable. Thus, CP55,940 does not produce reward-like effects in ICSS under these conditions.

Keywords: synthetic cannabinoid, intracranial self-stimulation, mice, withdrawal, tolerance, dependence, CP55,940.

1. Introduction

Cannabis sativa has been used both medicinally and recreationally for thousands of years (Mechoulam et al. 1991). The psychotropic effects of this plant are due mainly to its primary psychoactive constituent Δ^9-tetrahydrocannabinol (THC) (Mechoulam and Gaoni 1965; Martin-Santos et al. 2012). THC falls within the class of drugs known as cannabinoids, which draw their moniker from the cannabis plant. Cannabinoids are primarily defined by their ability to bind and activate cannabinoid receptor 1 (CB_1) (Herkenham et al. 1990; Matsuda et al. 1990) and cannabinoid receptor 2 (CB_2) (Munro et al. 1993). Although CB_1 is well known to play a predominant role in mediating the behavioral effects of THC and other cannabinoids and to modulate the rewarding effects of other classes of drugs (Rinaldi-Carmona et al. 1994; Ledent et al. 1999; Zimmer et al. 1999; Forget et al. 2005), emerging evidence indicates that CB_2 plays opposing roles in the reinforcing effects of cocaine and nicotine (Xi et al. 2011; Ignatowska-Jankowska et al. 2013; Navarrete et al. 2013).

In addition to THC, hundreds of synthetic cannabinoids vary in structure and bind and activate cannabinoid receptors (for review, see (Pertwee 2006)). These synthetic compounds were crucial for establishing the binding and distribution of cannabinoid receptors in brain (Devane et al. 1988; Herkenham et al. 1990). However, in recent years, synthetic cannabinoids such as CP-47,497 (Hudson et al. 2010), AM-2201 (Denooz et al. 2013), JWH-018, and JWH-073 (Brents and Prather 2014), emerged as new drugs of abuse. Synthetic cannabinoids are generally abused by smoking plant material imbued with these compounds in much the same manner as marijuana, and are readily available as preparations commonly referred to as “Spice” or “K2” among other brand names (Fantegrossi et al. 2014). Synthetic cannabinoids are often markedly more potent and/or efficacious than THC (Griffin et al. 1998). Moreover, toxicological information is limited, and

little is known about how these compounds affect brain reward circuitry *in vivo*. As synthetic cannabinoids have emerged as drugs of abuse (Maxwell 2014), further research is needed to characterize their pharmacology and toxicology. The impact of chronic exposure to nonclassical cannabinoids also remains to be determined.

Similar to other drugs of abuse, cannabinoids can evoke dopamine release in the nucleus accumbens (NAcc), a characteristic often indicative of drugs of abuse (Chen et al. 1993; Cheer et al. 2004). The NAcc is one node in a neural circuit known as the mesolimbic dopamine pathway, which consists of dopaminergic neurons that originate in the ventral tegmental area (VTA) and project to NAcc and more rostral targets such as prefrontal cortex (PFC). Intracranial self-stimulation (ICSS) of the medial forebrain bundle is one procedure that has been used to measure reinforced behavior mediated by the mesolimbic dopamine pathway (Carlezon and Chartoff 2007) and to assess abuse potential of drugs (Negus and Miller 2014). Although acute administration of synthetic cannabinoids generally suppresses ICSS (Arnold et al. 2001; Vlachou et al. 2003; Vlachou et al. 2005), the impact of repeated cannabinoid administration on ICSS has not been extensively studied but may be important. For example, repeated administration of mu opioid agonists evokes tolerance to their rate-decreasing effects and unmasks abuse-related ICSS facilitation (Altarifi and Negus 2011; Altarifi et al. 2012). Additionally, ICSS has been used to detect withdrawal-related anhedonia for some drugs of abuse such as cocaine, nicotine and morphine (Altarifi and Negus 2011; Stoker et al. 2012; Stoker et al. 2014).

In the present study we tested the hypotheses that (a) repeated administration of a synthetic cannabinoid will facilitate ICSS in a similar fashion as other abused drugs, and (b) spontaneous or antagonist-precipitated withdrawal in mice repeatedly administered cannabinoids will produce an anhedonia-like depression of ICSS similar to that produced by withdrawal from other abused

drugs. Because of the wide variety of synthetic cannabinoids and the ever changing composition of abused preparations, we chose to use a single, representative compound, CP55,940, to model acute and repeated effects of synthetic cannabinoids. Although CP55,940 has not emerged as a drug of abuse and has not been scheduled by the Drug Enforcement Agency, it has been extensively characterized in preclinical studies, and it is structurally similar to the abused and scheduled nonclassical cannabinoids CP47,497 and cannabicyclohexanol (Logan et al. 2012). Moreover, these compounds bind with similar affinity to CB_1 and CB_2 (Huffman et al. 2010; Atwood et al. 2011). Acute administration of CP55,940 depressed ICSS in rats (Arnold et al. 2001; Kwilasz and Negus 2012), but its consequences on ICSS following repeated administration are unknown.

In initial experiments, we examined the dose-response relationship and time course of the effects of acute CP55,940 administration on ICSS. Rimonabant was used to infer CB_1 involvement. We then tested whether the acute effects of CP55,940 on ICSS would undergo tolerance following repeated administration. Because cannabinoids are well established to alter motor function, such as catalepsy, we also assessed the relationship between catalepsy and ICSS measures during repeated administration of CP55,940 (Little et al. 1988). Catalepsy was selected as a concurrent endpoint because the behavior may confound the ability of the mice to engage in operant responding, and CB_1-mediated depression of ICSS may reflect non-specific disruption of behavior rather than an ICSS specific effect. Finally, we examined whether mice treated repeatedly with CP55,940 displayed signs of either spontaneous or precipitated withdrawal in the ICSS procedure.

1. Materials and Methods

2.1 Subjects

A total of 43 male C57Bl/6J mice were used (Jackson Laboratories, Bar Harbor, Maine). Mice were between 10 and 14 weeks of age at the start of each experiment and were individually housed and maintained on a 12 h light cycle, with lights on from 0600 to 1800 h, with free access to food and water. All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee (IACUC).

2.2 Drugs

CP55,940, rimonabant and cocaine HCl were obtained from the National Institute of Drug Abuse Drug Supply Program (Rockville, MD). CP55,940 and rimonabant were dissolved in a vehicle (VEH) consisting of 5% ethanol, 5% Emulphor-620 (Rhone-Poulenc, Princeton, NJ), and 90% 0.9% saline. Cocaine was dissolved in 0.9% saline.

2.3 Intracranial Self-Stimulation (ICSS)

2.3.1 Apparatus.

ICSS testing was conducted in eight mouse operant conditioning chambers (18 X 18 X 18 cm; Med Associates Inc., St. Albans, VT). Each chamber was equipped with a retractable lever located on one wall, LED stimulus lights over the lever, a chamber house-light, a tone-generator and an ICSS stimulator. The stimulator was connected to the electrode via bipolar cables routed through a swivel commuator and into the experimental chamber. Chambers were enclosed within sound- and light-attenuating chambers equipped with exhaust fans. Custom software was used to control manipulations in the operant chambers and to record data during training and testing sessions.

2.3.2 Stereotaxic Surgery

Surgical procedures for implanting electrodes in mice for ICSS studies were similar to those previously reported (Carlezon and Chartoff 2007; Wiebelhaus et al. 2014). Mice were anesthetized with isoflurane for implantation of bipolar twisted stainless steel electrodes (0.280 mm diameter and insulated except at the flat tips; Plastics One, Roanoke, VA) into the right medial forebrain bundle (2.0 mm posterior to bregma, 0.8 mm lateral from midline, and 4.8 mm below dura). The electrode was fixed to the skull with anchoring screws and dental cement. Mice were given acetaminophen (1-2 mg/ml) in their drinking water for one day before and five days after surgery. Training began one week after surgery.

2.3.4 Training

During initial training, lever-press responding under a fixed-ratio 1 (FR1) schedule produced both (a) delivery of a 0.5 s train of square-wave cathodal pulses (0.1 ms pulse duration) at a frequency of 141 Hz and amplitude of 150 □A and (b) 0.5 s onset of stimulus lights, house light, and tone cues. Responding during stimulation had no scheduled consequences. Amplitudes of stimulation were individually adjusted for each mouse to maximize response rates, and final amplitudes ranged from 45–300 μA. Training continued during daily 30–120 min sessions until response rates exceeded 30 responses per min for at least three days.

Once operant responding was established, mice were promoted to frequency-rate training as previously reported for mice (Wiebelhaus et al. 2014) and rats (Negus et al. 2010). Frequency-rate sessions were divided into multiple components, and each component consisted of 10 sequential frequency trials for presentation of a descending series of 10 stimulation frequencies (2.2-1.75 log Hz in 0.05 log increments). Each frequency trial began with a 10 sec time out period, during which behavior had no scheduled consequences. During the last 5 s of the time out period, the lever was extended, and non-contingent stimulations were delivered once per second at a given

frequency together with associated cues The time out period was followed by a 60 s response period when responding under the FR1 schedule produced brain stimulation at the specified frequency together with associated cues. After the 60 s response period, the lever was retracted, the stimulation frequency was decreased by 0.05 log units, and the next frequency trial began. Sessions consisted of three to five consecutive components per day, and current amplitudes were adjusted if necessary for each subject to maintain responding for at least three, and fewer than eight, stimulation frequencies at levels ≥ 50% maximal control rates (see Data Analysis).

Once these criteria were met, preliminary testing was initiated. Test sessions consisted of three baseline components followed first by a 30 min treatment interval and then by two test components. Data from the first baseline component for each test session were excluded from analysis. Data from the next two baseline components were averaged to generate baseline data for that test session, and data from the test components were averaged to generate test data. Mice were eligible for drug testing when the total number of stimulations per component during baseline varied by less than 20% on three consecutive days, and baseline and test numbers of stimulations per component differed by ≤ 20% in the absence of an injection or after treatment with vehicle injections. Brains were harvested from select mice, which met criteria throughout testing, and histological analysis was performed to verify of electrode placement into the lateral hypothalamus (Figure 31). Microscopy was performed at the VCU Department of Anatomy and Neurobiology Microscopy Facility, supported, in part, with funding from the NIH-NINDS Center core grant (5P30NS047463).

1.3.5 Dose-Effect Relationship of CP55,940

Once the training criteria were met, drug testing was initiated using dose-effect, time-course and repeated-dosing procedures. For antagonism studies, a single dose of CP55,940 (0.03-

1.0 mg/kg, s.c. 30 minutes prior to test components) was administered alone or 15 min after rimonabant (3-30 mg/kg). The effects of cocaine (10.0 mg/kg, i.p. 10 min prior to testing) were also tested as a positive control (Figure 32). Test sessions were separated by at least 72 hr.

2.3.6 Time Course of CP55,940.

The procedures described above were modified to assess the onset and duration of effects produced by CP55,940. Test sessions consisted of three baseline components followed first by a 5 min treatment interval and then by pairs of test components beginning 5 min, 30 min, 2 hr, 4 hr and 8 hr after injection. Mice were removed from the test chamber between the last four pairs of test components. If necessary, drug effects were also evaluated after 24 and 48 hr. The time course of vehicle injection was tested first. If the number of stimulations per component varied ≤ 20% for all test components from 5 min to 8 hr, then an identical procedure was used to evaluate effects of 0.3 and 1.0 mg/kg CP55,940. Two different groups of mice were used for each dose in the time course studies. Thus, each group had a corresponding vehicle time course for comparison.

2.3.7 Repeated Administration of CP55,940

To test the effects of a fixed dose of CP55,940 administered repeatedly, two groups of mice were assessed in three phases, each of which lasted for seven days. On each day of Phases 1 and 2, test sessions consisted of three baseline components followed first by treatment interval and then by two test components. Injections were administered during the treatment interval, 30 min before initiation of the test components. In addition, mice were tested for catalepsy (see below) immediately before the injection and again approximately 20 min after completion of the test components (75 min after the injection).

The first phase of testing consisted of seven consecutive days of vehicle testing using the procedure described above. This phase established a baseline for comparison to phases 2 and 3 and permitted assessment of stability of responding during daily vehicle injections. If the number of stimulations per component during baseline components varied ≤ 20%, across days, and if the number of baseline and test stimulations per component varied ≤ 20% on each day, then mice were advanced to subsequent phases. In phase two, the mice were divided into separate groups. One group continued receiving daily injections of vehicle, whereas the second group received daily injections of 0.3 mg/kg CP55,940. In phase 3, treatment with CPP55,940 terminated, and only baseline components were conducted to probe for evidence of spontaneous withdrawal and to investigate whether or not mice would return to pre-drug baselines.

Precipitated withdrawal experiments were conducted in a third group of mice in three phases as described above with the following procedural differences. Four h after the final injection of phase 1, mice were injected with 10 mg/kg rimonabant to examine its effects on ICSS before CP55,940 exposure. After two or three days of subsequent vehicle tests to allow for washout of rimonabant, mice were given daily injections of 0.3 mg/kg CP55,940 (Phase 2). On day 7 of phase 2 mice were given a second test with rimonabant (10 mg/kg, 10 min i.p., 4h after CP55,940) to precipitate withdrawal.

2.3.8 *Data Analysis.*

All data were analyzed using GraphPad Prism 6.0 software. The primary dependent variables were the number of stimulations per min for each frequency and the number of stimulations per component across all frequencies as described previously (Negus and Miller 2014; Wiebelhaus et al. 2014). These data were then evaluated using two separate approaches. First, data for each frequency trial during baseline and test components were expressed as percent Maximum

Control Rate (%MCR), with maximum control rate defined as the maximum average rate observed at any frequency during baseline components for that mouse on that day. %MCR data were averaged across mice to generate the frequency-rate curves that were assessed using repeated-measures two-way ANOVAs (treatment x frequency) between baseline and treatment curves for each drug/dose tested. A significant ANOVA was followed by the Holm-Sidak test, and the criterion for significance was $p<0.05$.

In the second approach, the average total number of stimulations per test component was divided by the average number of stimulations per baseline component for each mouse on each day, and multiplied by 100, to produce percent baseline stimulations (% baseline stimulations). These data were averaged across mice for each treatment and compared with one-way ANOVAs (Wiebelhaus et al. 2014). A significant ANOVA was followed by Dunnett's *post hoc* test to compare treatment groups with VEH controls.

Data from repeated-treatment studies were analyzed using both % MCR and % baseline stimulations measures. For tolerance studies each day in phase 2 was compared within each group to each other day as well as to average data from phase 1. Additionally, % baseline stimulations were also analyzed between groups by day. Selected frequency-rate curves from phase 1 day 7 (i.e. pre-drug), and days 1, 2 and 7 from phase 2 were used to assess both tolerance across days and the acute effect of CP55,940 each day. Precipitated withdrawal studies compared the frequency-rate curves between baselines, CP55,940 tests, and rimonabant tests in phase 1 day 7 and phase 2 day 7.

2.4 Catalepsy

2.4.1 Procedure.

Catalepsy was measured during 60 s test periods. At the start of each test period, the forepaws of the mouse were placed on a metal bar raised 4.5 cm from a metal platform. If a mouse removed its forepaws from the bar, the forepaws were replaced up to four times or until the testing period ended, whichever occurred first. The total time the mouse retained its forepaws on the bar was recorded. During the spontaneous and precipitated withdrawal experiments, catalepsy was measured immediately after ICSS baseline and test components. Approximately 75 min lapsed between these measurements during ICSS studies, so the same interval was used in control experiments without ICSS.

2.4.2 Data Analysis.

Catalepsy data are expressed as the change from baseline measurements after a 75 min pretreatment and analyzed utilizing two-way ANOVA. To determine whether the expression of catalepsy correlated with rate-decreasing effects of repeated dosing with CP55,940, a Pearson correlation was conducted between the change from baseline in catalepsy versus the % baseline stimulations.

3. Results

3.1 Potency, Time Course and Rimonabant Antagonism of CP55,940 Effects on ICSS

Whereas cocaine (10 mg/kg) facilitated ICSS (Figure 31), CP55,940 (30 min pretreatment) produced dose-related reductions in ICSS. The % baseline stimulations measure shows that CP55,940 (0.3-1.0 mg/kg) depressed ICSS with no evidence for facilitation at any dose (Figure 27A, ($F(2.640, 18.89) = 40.1$) $p < 0.001$). The ED_{50} (95% confidence limits) of CP55,940 to produce rate-decreasing effects was 0.15 (0.12-0.18) mg/kg. Similarly, CP55,940 produced dose-dependent rightward and downward shifts in the ICSS frequency-rate curve (Figure 27B, ($F(9, 63) = 85.9$, $p < 0.001$). Finally, time-course studies revealed that 0.3 and 1.0 mg/kg CP55,940 decreased ICSS within 5 min, and these rate-decreasing effects persisted for up to 8 h ($F(18, 132) = 20.27$, $p < 0.001$).

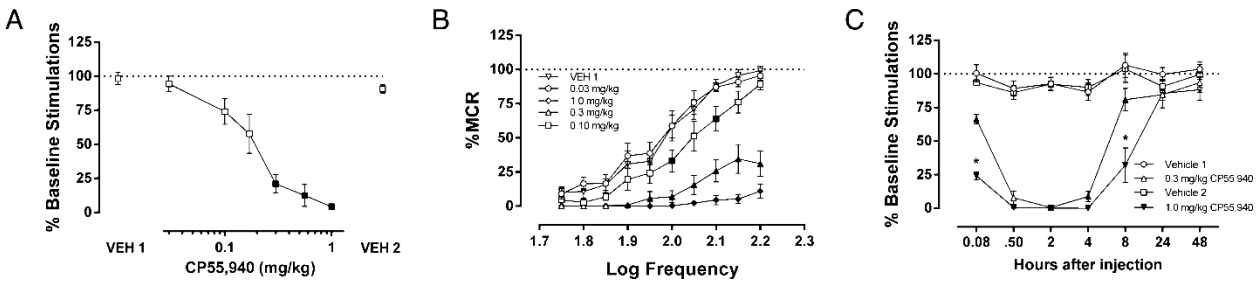

Figure 27. Acute administration of CP55,940 suppressed ICSS through a CB_1 receptor mechanism of action. **A.** (%Baseline stimulations) and **B.** (%MCR). CP55,940 dose-dependently decreased ICSS. n=8; filled squares indicate $p<0.05$ vs. VEH 1, **C.** CP55,940-induced ICSS depression persisted for up to 8 h (n=6-7, filled squares indicate $p<0.05$ vs. respective vehicle control, *$p<0.0001$ 0.3 mg/kg vs. 1.0 mg/kg CP55,940).

Whereas rimonabant (3.0-30.0 mg/kg) administered alone had no effect on % baseline stimulations (Figure 28 and Figure 32), it dose-dependently prevented the depressive effects of CP55,940 (1.0 mg/kg) on ICSS (Figure 28A: $F(2,12) = 104.7$, $p < 0.001$). Analysis of the frequency-rate data indicated no effect of rimonabant alone (Figure 28B: $F(18, 108) = 1.131$, $p = 0.333$), and a dose-dependent reversal of CP55,940-induced suppression of ICSS (Fig 28C: $F(27, 162) = 18.4$, $p < 0.001$).

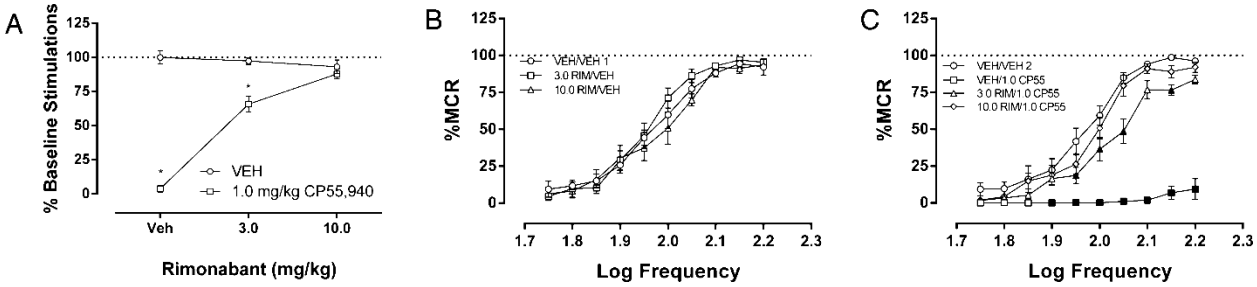

Figure 28. A. The CB_1 antagonist rimonabant (3.0 or 10.0 mg/kg) did not affect ICSS when administered alone, but blocked the rate-decreasing effects of CP55,940 (n=7, filled squares indicate $p<0.05$ VEH vs. 1.0 mg/kg CP55,940, *$p<0.0001$ vs. 10.0 mg/kg rimonabant) **B**. Frequency-rate analysis of rimonabant alone and vehicle test revealed no significant difference (n=7). **C.** CP55,940 (1.0 mg/kg) suppressed ICSS, which was prevented by rimonabant (n=7, filled squares indicate $p<0.05$ vs vehicle 2).

3.2 Effects of Repeated CP55,940

The average % baseline stimulations for each mouse during seven days of vehicle injections was used for comparison to assess effects of phase 2 treatments with vehicle or different CP55,940 doses (seven day averages (±2.14 SEM) for the number of baseline stimulations per component: Group 1: 97.73 (±2.14), Group 2: 102.49 (±1.57), Group 3: 99.11 (±2.52)). Figure 29A shows effects of repeated vehicle or 0.3 mg/kg CP55,940 on % baseline stimulations during phase 2 ($F(14, 91) = 3.3$, $p < 0.001$)). Repeated treatment with vehicle during phase 2 did not alter ICSS. However, the first injection of CP55,940 (0.3 mg/kg) significantly reduced ICSS to a similar degree as in the dose-effect study (see Figure 27A). Tolerance to rate-decreasing effects developed by day 3 in both CP55,940-treated groups, and on day 5, both groups of CP55,940-treated mice no longer displayed differences from the vehicle-injected mice. Repeated treatment with vehicle during phase 2 also produced no change in frequency-rate measures of ICSS (Figure 29B; $F(27, 108) = 0.74$, $p = 0.81$). The CP55,940-treated groups showed an initial suppression of ICSS on day 1 and partial recovery of ICSS occurred on later days (Figure 29C; $F(27, 135) = 3.4$, $p < 0.0001$; Figure 29D; $F(27, 135) = 1.784$, $p < 0.05$).

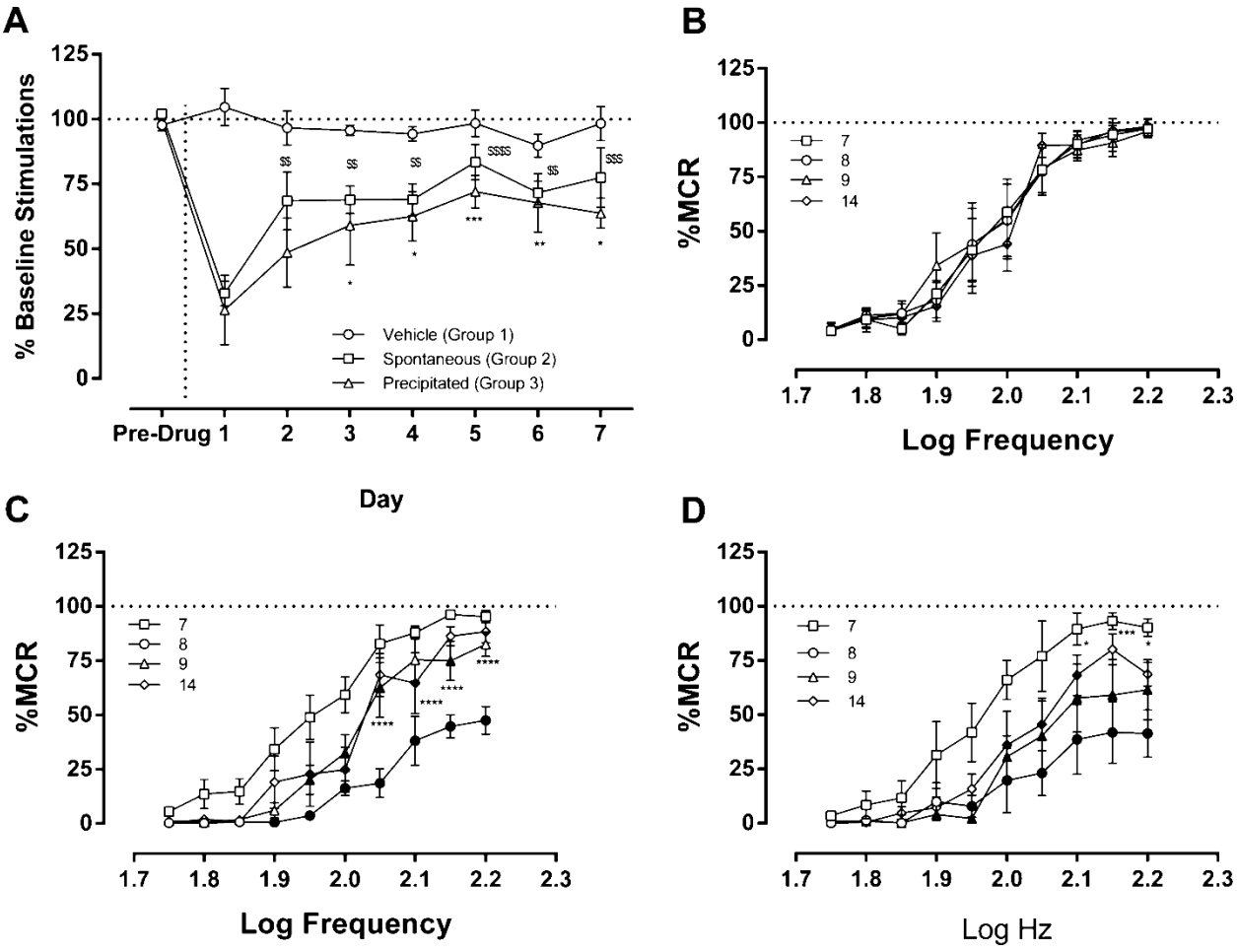

Figure 29. Partial tolerance developed to a fixed dose of CP55,940 (0.3 mg/kg). **A.** Tolerance developed to the rate-decreasing effects of CP55,940 by day 2 in CP55,940 Group 1 (n=6, $$p<0.01, $$$p<0.001, $$$$p<0.0001 vs. day 1) and by day 3 in CP55,940 Group 3 (n=6, *p<0.05, **p<0.01, ***p<0.001 vs. day 1, although rates of responding never returned to vehicle levels within either group. **B.** The response rates for the repeated vehicle group remained ±20% of their phase 1 average, indicating no effect of repeated injections on ICSS (n=5).**C, D.** Frequency-rate analysis revealed similar pattern of tolerance in mice receiving repeated administration of CP55,940 (CP55,940 Group 2 and 3 (filled squares p<0.05 vs. pre-drug, ****p<0.0001 day 1 vs. day 7).

Repeated treatment with 0.3 mg/kg CP55,940 also increased catalepsy in ICSS mice as well as in a separate group of mice that did not undergo ICSS training and testing. (Supp. Figure 34A, $F(14, 105) = 2.693$, $p < 0.01$). Catalepsy was assessed after baseline ICSS and after CP55,940 administration to assess the role of this motor behavior. The cataleptic effects of CP55,940 were greatest on day 1 of treatment and reduced by day 2, but significant catalepsy persisted throughout the seven days of repeated administration. There was no correlation between the degree of catalepsy and the degree of ICSS suppression (Supp. Figure 34B; $r = 0.07$, $p=0.69$).

Termination of vehicle or CP55,940 treatment did not alter ICSS during the subsequent seven days (Figure 30A; interaction day vs group $F(40, 280) = 0.8$, $p = 0.74$). In addition, administration of 10 mg/kg rimonabant did not depress ICSS when it was administered before or after repeated treatment with CP55,940. Figure 30B shows that rimonabant did not alter ICSS as measured by % baseline stimulations in this group of mice ($F(18, 90) = 0.80$, $p = 0.69$). Figure 30C shows the effects of 10 mg/kg rimonabant on full frequency-rate curves before CP55,940 treatment ($F(18,90) = 0.80$, $p = 0.69$). The final dose of 0.3 mg/kg CP55,940 produced acute suppression of ICSS relative to the pre-drug baseline, and rimonabant blocked this suppression (Figure 30D, $F(18, 90) = 2.0$, $p< 0.05$).

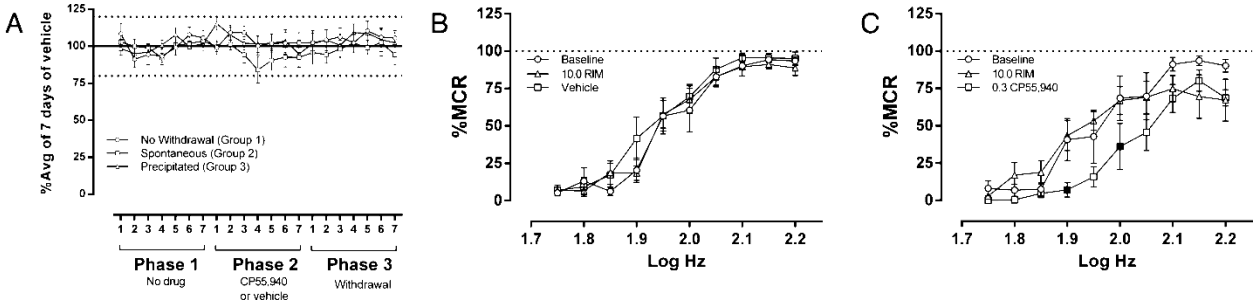

Figure 30. There was no evidence of spontaneous or precipitated withdrawal following 7 days of once daily injections of 0.3 mg/kg CP55,940. **A.** Basal ICSS responses for each phase of testing (tests were performed prior to injections of vehicle or drug).Throughout the 21 days of testing, each group did not deviate ±20% on during basal testing. **B.** Rimonabant (10.0 mg/kg, i.p.) did not affect ICSS in mice treated repeated with either vehicle or CP55,940 (n=6) **C.** Frequency-rate curves on the same days revealed no effect of rimonabant after 7 days of vehicle on phase 1 day 7 (n=6). **D.** CP55,940 (0.3 mg/kg) administered on seven consecutive days suppressed ICSS. Rimonabant (10.0 mg/kg) 4 hours after the final CP55,940 injection returned responding to baseline levels, consistent with pharmacological blockade of CB_1 (n=6, filled square indicate $p<0.05$ vs baseline).

4. Discussion

Acute administration of CP55,940 dose-dependently and time-dependently depressed ICSS in mice. The observation that rimonabant prevented the rate-decreasing effects of CP55,940 indicates that these effects were CB_1 receptor mediated. The fact that rimonabant given alone did not alter ICSS indicates that CB_1 receptors play a negligible role in basal responding in this ICSS procedure. Partial tolerance developed after repeated exposure to a fixed dose of CP55,940, but there was no evidence to suggest that this tolerance to rate-decreasing effects of CP55,940 unmasked expression of abuse-related rate-increasing effects. Moreover, neither spontaneous nor rimonabant-precipitated withdrawal altered ICSS in mice treated repeatedly with 0.3 mg/kg CP55,940, suggesting that this regimen of CP55,940 treatment was not sufficient to produce dependence. Finally, catalepsy did not correlate with reduced ICSS after exposure to CP55,940. Taken together, these results indicate that CP55,940 did not produce reward-like effects in ICSS after either acute or repeated administration.

4.1 Acute effects of CP55,940 in ICSS

Acute administration of CP55,940 suppressed ICSS in mice with a potency approximately 57 (35-95)-fold greater than that of THC (Wiebelhaus et al. 2014). The observed difference in potency is consistent with the affinities of THC and CP55,940 for CB_1. Reported *in vitro* K_i values for THC are approximately 40 nM, whereas CP55,940 Ki is approximately 0.9 nM, reflecting a difference in affinity of 45 fold for the CB_1 receptor (Compton et al. 1993). Potencies between THC and CP55,940 *in vivo* range from 4- to 15-fold in catalepsy, tail withdrawal, and rectal temperature assays (i.v. route of administration) in male ICR mice (Compton et al. 1992) and up to 82-fold in a drug discrimination procedure (i.p. route of administration) in male C57BL6/J mice (McMahon et al. 2008). Previous studies examining the acute effects of CP55,940 found that a

dose range of 0.01-0.05 mg/kg did not affect ICSS, but doses of 0.1 mg/kg and higher depressed ICSS in rats (Arnold et al. 2001; Mavrikaki et al. 2010; Kwilasz and Negus 2012). The present study represents the first publication reporting the effects of CP55,940 on ICSS in mice, and we found that a similar dose range reduced ICSS in this species. Importantly, there was no evidence for ICSS facilitation at low CP55,940 doses that did not suppress ICSS in mice, consistent with previous studies investigating synthetic cannabinoids in rat ICSS (Arnold et al. 2001; Mavrikaki et al. 2010; Kwilasz and Negus 2012). Although facilitation of ICSS by THC has been reported previously in rats (Gardner et al. 1988; Katsidoni et al. 2013), it should be noted that facilitation generally occurs at low doses in select strains of rats (Lewis and Sprague-Dawley) in a subset of published studies, and the magnitude of facilitation is relatively small compared to that produced by psychomotor stimulants such as cocaine (for review, see Negus and Miller 2014). Furthermore, THC attenuates ICSS in mice. Given the failure of CP55,940 to produce evidence of ICSS facilitation even at doses as low 0.03 mg/kg, the results of the present extend the range of conditions under which cannabinoids fail to facilitate ICSS in rodents.

Whereas rimonabant completely prevented CP55,940-induced depression of ICSS, this drug given alone did not alter ICSS. Previous studies in rats and mice also found that ICSS was not altered by CB_1 receptor antagonist doses sufficient to block effects of exogenous cannabinoids (Vlachou et al. 2005; Vlachou et al. 2006; Kwilasz and Negus 2012; Katsidoni et al. 2013). These findings suggest that endocannabinoids acting at CB_1 receptors do not tonically modulate neural substrates that mediate ICSS.

4.2 Tolerance to the rate-decreasing effects of CP55,940

In the present study, the depressive effects of 0.3 mg/kg CP55,940 on ICSS underwent tolerance by the second day of treatment. While this tolerance persisted for the remaining seven

days of drug administration, tolerance was not complete, as CP55,940 continued to attenuate ICSS. For comparison, complete tolerance developed to THC-induced depression of ICSS in rats treated for 22 days with an escalating regimen of THC doses (Kwilasz and Negus 2012), but no tolerance developed to depression of ICSS by repeated treatment with the synthetic cannabinoid WIN55,212-2 (Mavrikaki et al., 2010). Although assessment of rightward shifts in the dose response relationship to quantify the magnitude of tolerance were not conducted, these apparent discrepancies in tolerance development to a single dose of drug may be related to the rank order of efficacies of these cannabinoids at CB_1 receptors (WIN55,212-2>CP55,940>THC) (Breivogel et al. 1998). Similarly, the extent of antinociceptive tolerance to mu opioid agonists was found to be inversely related to efficacy of the agonists at mu receptors (Yaksh 1992; Duttaroy and Yoburn 1995). More generally, it appears that low- vs. high-efficacy ligands occupy higher proportions of receptors to produce equivalent acute effects, down-regulate a higher proportion of receptors during chronic treatment, and are more sensitive to reductions in the density of functional receptors produced by that downregulation.

5. Conclusions

The findings in the present study indicate little evidence for abuse potential and dependence for CP55,940. Tolerance developed quickly but incompletely to the rate-decreasing effects of CP55,940 on ICSS. The bulk of studies investigating the effects of synthetic cannabinoids on ICSS examined acute drug administration, and only one study of which we are aware examined repeated administration of a synthetic cannabinoid in rats (Mavrikaki et al. 2010). Thus, the present body of work represents the first study to examine tolerance and dependence of a synthetic cannabinoid in a mouse ICSS procedure. Although there was no evidence for spontaneous or rimonabant-precipitated withdrawal, the depressive effects of

CP55,940 on ICSS showed partial tolerance following repeated administration. It is reasonable to suspect these findings could be extended to other bicyclic cannabinoids which have a history of abuse such as CP47,497 (Papanti et al. 2013; Koller et al. 2014). Overall, these experiments reveal the greatly increased potency of synthetic cannabinoids and their potentially detrimental effects on brain reward following acute or repeated administration.

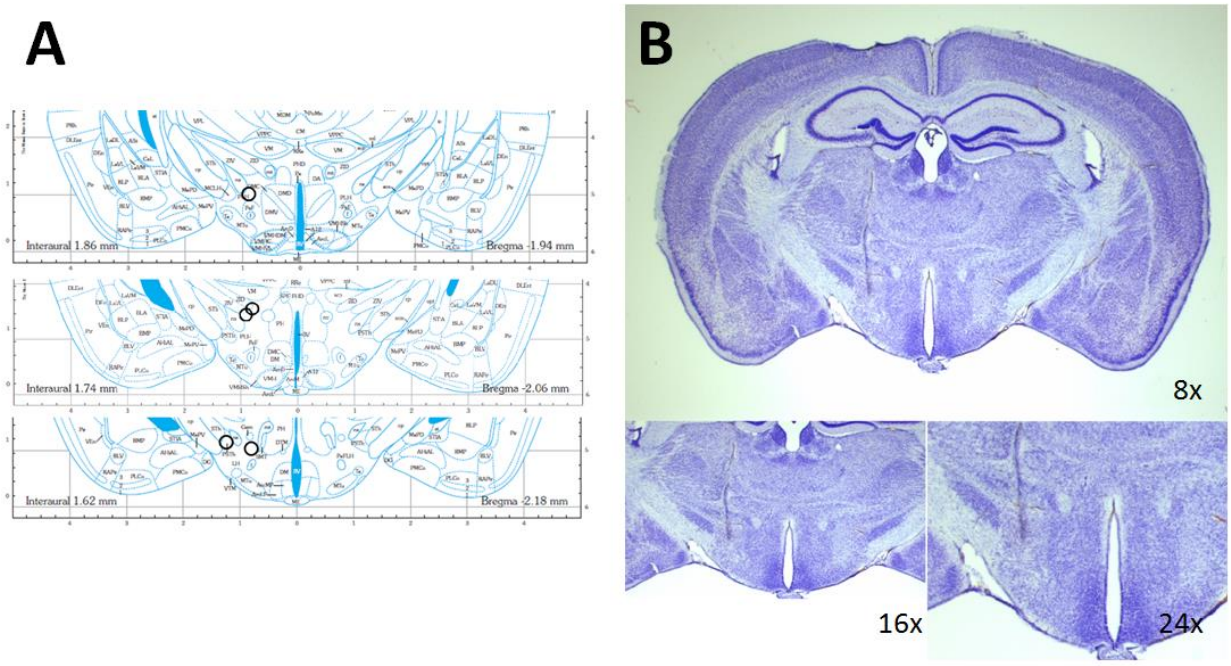

Figure 31. Select mice that completed at least one experiment were anesthetized with 2-3% isoflurane and humanely sacrificed via cervical dislocation. Whole brains were harvested and immersed in 8-10 ml of 10% formalin for 7-10 days to allow for tissue fixation. The brains were sliced in 50 μm sections using a Leica VT1000S Vibratome, mounted on Superfrost+ slides (ThermoFisher Scientific, Waltham, MA), and allowed to dry overnight. The sections were stained for Nissl with cresyl violet and imaged using a Zeiss Discovery V20 Stereo Zoom microscope. **A.** Electrode tips are localized to the lateral hypothalamus in mice which qualified for testing in ICSS utilizing the requirements detailed in the Methods section. **B.** A representative section from a mouse showing a partial electrode tract as well as localization of the tip (8x, 16x, and 24x magnification).

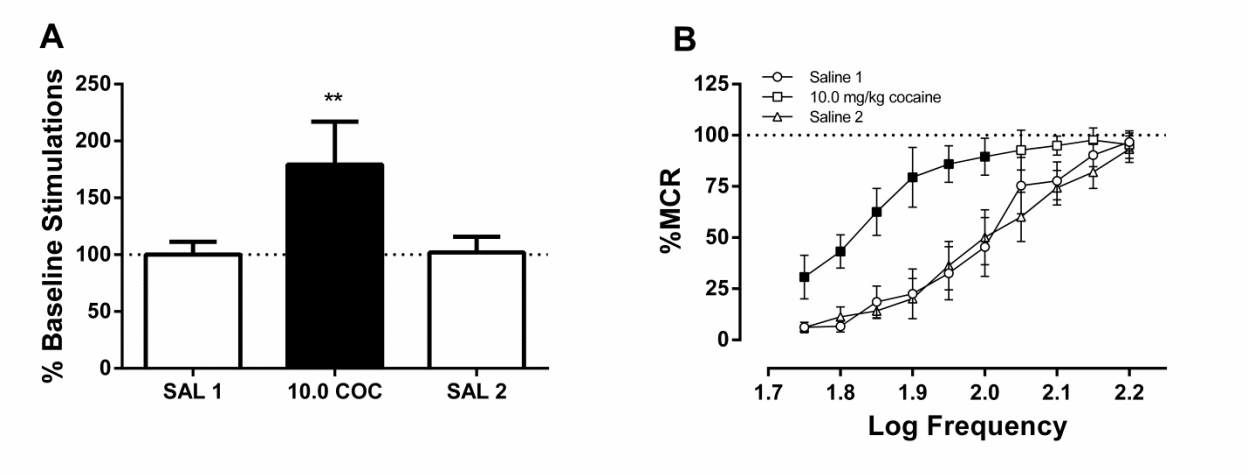

Figure 32. Cocaine HCl (10 mg/kg) served as a positive control to demonstrate ICSS-facilitating effects in mice. Cocaine increased responding in both **A. %**baseline stimulations and **B.** frequency-rate curves ($F(1.051, 5.257) = 19.20$, $p < 0.01$, n=6, *$p < 0.05$ vs. saline 1, filled squares $p<0.05$ vs. baseline).

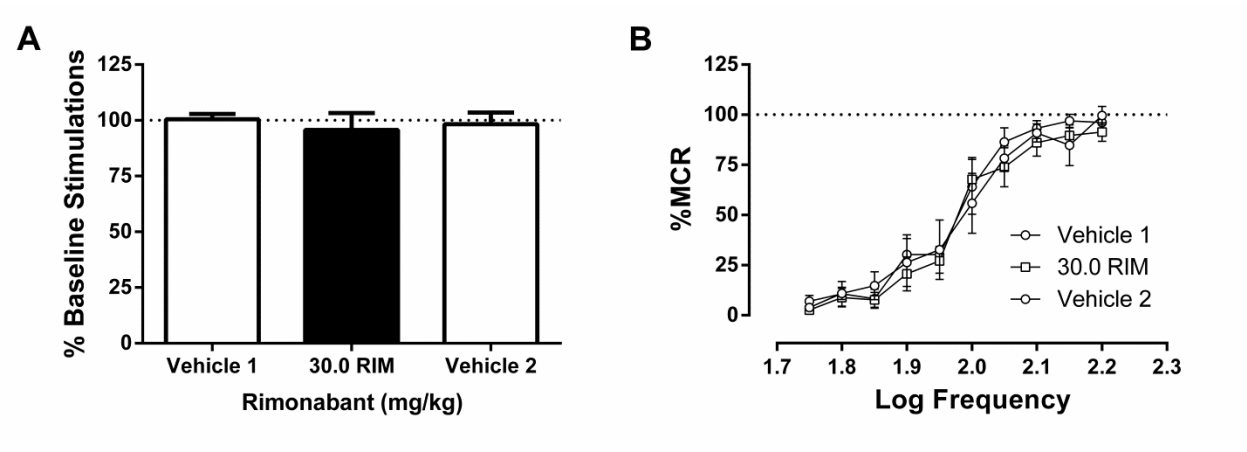

Figure 33. A single, high dose of rimonabant (30 mg/kg, 45m s.c.) did not suppress ICSS in **A.** %baseline stimulations ($F(1.481, 5.925) = 0.23$, $p=0.74$) or **B.** frequency-rate ($F(18, 72) = 1.125$, $p=0.3500$).

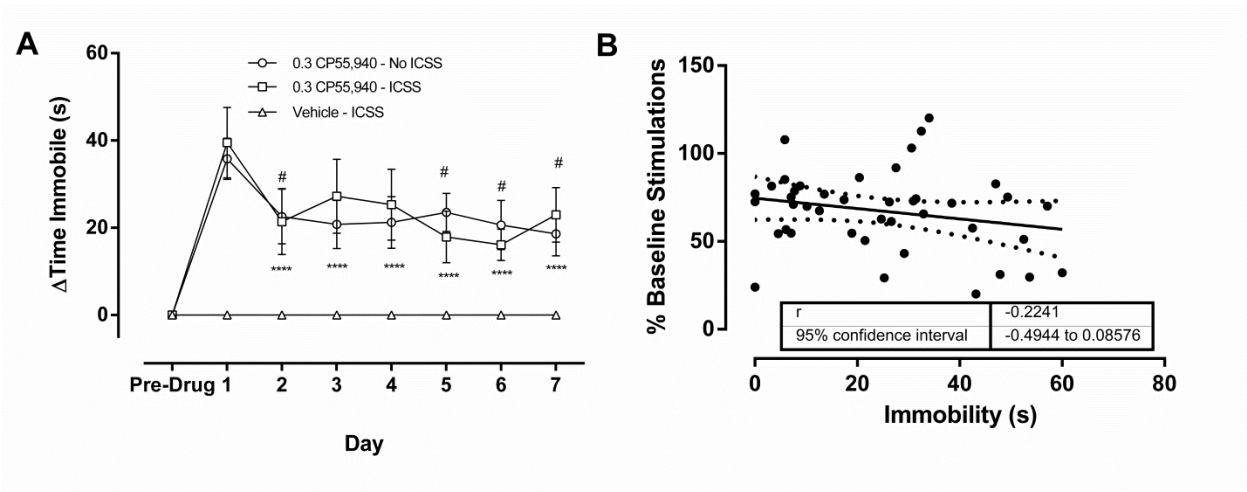

Figure 34. A. Tolerance developed to the cataleptic effects of CP55,940 in mice implanted with electrodes in the medial forebrain bundle ($F(14, 105) = 2.69$, $p < 0.05$, n=6-8, #p<0.05 vs. day 1 for 0.3 CP55,940 – ICSS, ****p<0.0001 vs. day 1 for 0.3 CP55,940 – no ICSS). In vehicle and 0.3 mg/kg CP55,940 – ICSS groups, each mouse was assessed for catalepsy following ICSS baseline and test passes, with 75 min elapsing between measurements. The 0.3 mg/kg CP55,940 – no ICSS group served as a control to determine if ICSS testing prior to catalepsy testing had any effect on the development of tolerance to the cataleptic effects of CP55,940. **B.** No significant correlation ($r = -0.22$, $p = 0.15$) was found between ICSS depression (30-60 min after injection) and catalepsy (75 min following injection).

Chapter 4: Discussion

4.1 Synthetic cannabinoids remain a prominent public health concern

As more case reports emerge detailing the deleterious effects of SC use, there is a great need to discover the mechanism by which they engender their dangerous and potentially deadly effects. Their structures vary considerably; thus the potential for toxic metabolites distinct from those found in marijuana is high. Few studies have examined the various metabolites in blood or urine following SC ingestion, and potential toxicological effects of these metabolites have not yet been elucidated. Another potential mechanism for the elevated risk of use lies within the pharmacological properties of the abused SC. As has been detailed in previous sections, SCs are generally more potent and efficacious at the CB_1 receptor, and health complications resulting from SC ingestion are generally congruous with peripheral and central CB_1 expression. Thus, if enhanced, *in vivo* activation of CB_1 is one of the determining factors to cause serious health complications, there is a need for a way to quickly ascertain efficacy to produce CB_1 activation. Additionally, the abuse-related effects of enhanced CB1 efficacy are not well understood, especially under repeated dosing conditions. Here, we developed a model utilizing receptor theory as a framework to use mice which possess 100%, 50%, and 0% CB_1 expression in a model of cannabimimetic activity. The compounds A-834,735D, WIN55,212-2, CP55,940, JWH-073, CP47,497, and THC were selected, based upon previously published results and personal communications, to span the efficacy continuum and assess stratification by efficacy *in*

vivo. Abuse-related effects of a representative high-efficacy cannabinoid (CP55,940) were tested employing the ICSS procedure after both acute and repeated administration. Finally, studies of agonist-stimulated [^{35}S]GTPγS experiments corroborated our *in vivo* results, although the observed rank order of efficacy did not entirely agree with previous reports.

4.2. Theoretical predictions versus agonist-stimulated [^{35}S]GTPγS binding in high and low receptor conditions

To elucidate whether CB_1-mediated G-protein activation correlated with agonist dose ratios in WT and HET mice, agonist-stimulated [^{35}S]GTPγS binding studies were conducted in WT, HET, and KO tissue. Agonist-stimulated [^{35}S]GTPγS binding assesses ligand potency and efficacy to affect the exchange of GTP for GDP at the G-protein, the first step in the canonical GPCR signal transduction pathway. Although the ligands for this body of work were selected utilizing the available body of literature, not all of these drugs had been tested under the same experimental conditions. WIN55,212-2, CP55,940, JWH-073, and THC were all tested in one study (Griffin *et al*, 1998), and the rank order generated for the experiments conducted here is in accordance with those results. Unpublished results comparing A834,735D versus CP55,940 (Thomas and Wiley, 2014) and CP47,497 and CP55,940 (unpublished results, Lichtman lab) also agree with our *a priori* rank order efficacy prediction. Additionally, the literature lacks a rigorous assessment of potential off-target G-protein stimulation utilizing CB_1 KO tissue despite known examples of non-CB_1, non-CB_2 mediated WIN55,212-2 stimulated [^{35}S]GTPγS binding. Therefore, a series of binding experiments were performed in high (cerebellum) and low (spinal cord) CB_1 receptor density tissues to assess the loss of efficacy as it relates to total receptor expression in WT and HET samples.

Radioligand binding with [^{3}H]SR141716A confirmed high CB_1 expression in cerebellum and low expression in spinal cord in WT mice, and that HET mice displayed roughly 50% expression in each case. No specific binding was detected in KO samples, confirming the selectivity of rimonabant. As discussed in the introduction, the E_{max} in each tissue should vary with receptor expression such that high expression will yield high E_{max} values and low expression will yield low E_{max} values. All ligands stimulated [^{35}S]GTPγS binding to a greater degree in cerebellum relative to spinal cord. In cerebellum, WIN55,212-2 elicited the highest E_{max} values, but unlike the other agonists tested, it also stimulated G-protein activation in KO tissue consistent with previous findings (Monory *et al*, 2002). To quantify and express only CB_1-mediated G-protein activity, the KO stimulation was subtracted from WT and HET stimulation for all drugs in cerebellar tissue. With this manipulation, A-834,735D, WIN55,212-2, CP55,940, JWH-073, and CP47,497 produced similar maximum stimulation in WT samples whereas THC achieved a relatively lower maximum. In HET samples, the predicted 50% decrease in Emax was not observed for the three highest efficacy ligands (A-834,735D, WIN55,212-2, and CP55,940), but rather a 20% decrease was observed. This smaller than expected decrease is likely indicative of a receptor reserve to produce G-protein activation, which is somewhat surprising. The stoichiometry of the receptors and G-proteins is important to consider when interpreting these results. Cannabinoids possess a low degree of efficiency to activate G-protein, with the full agonist WIN55,212-2 stimulating GDP-GTP exchange at three G-proteins per receptor activated indicating an amplification factor of 3 (Sim *et al*, 1996b). It is unknown if this amplification factor is a general property of cannabinoid receptors or if it is ligand-specific. In spinal cord, the expected 50% reductions were observed for all ligands except for THC, which did not have detectable efficacy to stimulate any GDP-GTP exchange in HET tissue. This result indicates low

receptor reserve conditions similar to apparent low receptor reserve for some the *in vivo* effects, though it should be noted the basal activity of spinal cord was nearly twice that of cerebellum (~4000 counts versus ~2000). This basal activity is likely not due exclusively to CB_1 receptors alone but rather reflects the sum of the constitutive activity of all GPCRs present in the sample aside from purinergic activity. Whether G proteins are pre-coupled to their receptors remains contentious, though if this is this case, then the high basal activity in the spinal cord might also reflect sequestration of a portion of the G-proteins by non-CB_1 receptors, functionally reducing the overall ability of the system to respond to cannabinoid ligands. G-proteins may also affect the conformation of the receptor they interact with, thereby also potentially affecting the affinity of the ligand for the receptor. Kenakin illustrates this by demonstrating the discrepancy between the use of agonist versus antagonist radioligands to measure affinity under conditions where $[G] < [R_T]$, $[G] = [R_T]$, and $[G] >>> [R_T]$ (Kenakin, 1997). Presuming an agonist favors the G-protein bound state, low [G] would reduce the potency of agonists while high [G] would increase potency. While the EC_{50} values reported here do vary somewhat between brain regions for some agonists, no discernable pattern emerged to suggest this to be the case. Overall, the results were expected in both cerebellum and spinal cord.

4.3 Differential sensitivity of catalepsy, hypothermia, and antinociception in the cumulative dosing CB_1 efficacy determination model

The cumulative dosing cannabinoid triad procedure revealed differential sensitivity of each endpoint to a 50% reduction in CB_1 receptor expression. The hypothesis that efficacy would vary inversely with the potency shifts measured between WT and HET mice was tested, with KO mice serving to detect any non-CB_1 effects for each measure. As an ancillary goal, this procedure was designed as a high throughput method to test *in vivo* efficacy of novel, putative

cannabimimetic ligands. Catalepsy, hypothermia, and antinociception are likely mediated by distinct CNS regions, which may account for observed differences among the measures.

Antinociception was the most sensitive of the three triad measures to a 50% reduction in CB_1 expression, and it was the only measure to display graded downward shifts in the dose response curve. The choice of a 10 second cutoff for tail withdrawal latency avoids tissue damage that could confound results, especially in repeated testing situations such as time course or cumulative dosing studies. Animal welfare concerns also limit the duration of testing, as tissue damage may occur at temperatures above 52°C or if the cutoff extends beyond ten seconds. Though raising the time limit may better differentiate between high efficacy ligands it would preclude the use of repeated testing to avoid tissue damage. The intensity of the stimulus (i.e. temperature of the water bath) is another variable which can be manipulated. As in the case of the 10 second time limit, the upper limit of the temperature was chosen to allow repeated testing and avoid confounding tissue damage. However, an alternative approach to differentiate between high-efficacy cannabinoids ligands such as A-834,735D and WIN55,212-2 would be to raise the stimulus intensity to elicit antinociception (e.g., increase the temperature of the hot water noxious stimulus). Although drugs of moderate efficacy may begin to resemble THC (i.e. no effect in HET mice), resolution would be gained at the top of the efficacy continuum. Reducing the stimulus intensity by reducing the temperature should have the opposite effect, in essence causing greater differentiation between low-efficacy compounds but less differentiation between moderate and high-efficacy compounds.

The tail-flick response (D'Amour and Smith, 1941) is a spinal reflex conducted primarily by fast conducting Aδ fibers from the source of the noxious insult to the dorsal horn. From there, the signal is transmitted along the spinothalamic tract to the thalamus, and parallel fibers project

along the spinomesecephalic tract to the PAG. Descending projects from the PAG through the rostroventromedial medulla to spinal neurons provide top-down control of the reflex (Morgan *et al*, 2008). Importantly, CB_1 is expressed in the dorsal root ganglion (Bridges *et al*, 2003), the dorsal horn (Farquhar-Smith *et al*, 2000), and the PAG (Herkenham *et al*, 1990) with appreciable abundance, although its expression in the PAG is somewhat higher than in spinal cord. When utilizing CB_1 HET mice, expression was 50% of that observed in WT mice. Given that CB_1 expression is already relatively low, receptor reserve may have been depleted sufficiently to detect downward shifts in the dose-effect relationship in HET mice. Indeed, the low–efficacy agonist THC did not produce dose-dependent increases in tail withdrawal latency in HET mice while doing so with relatively low potency in WT mice compared to the other agonists tested. CP47,497 and JWH-073 did elicit dose-dependent increases in tail withdrawal latency in HET mice but did not achieve above a 50% effect to allow ED_{50} potency comparisons. CP55,940, WIN55,212-2, and A-834,735D all achieved maximal effects in HET mice though they did so with varying potency. The final efficacy rank order was similar to the hypothesized order (A-834,735D ≥ WIN55,212-2 > CP55,940 > JWH-073 ≥ CP,47497 > THC), though the decline in potency was much steeper when compared to hypothermia. This finding suggests the tail withdrawal assay under these conditions has the highest efficacy requirement of the three endpoints, though this sensitivity may be adjusted as discussed above.

Hypothermia displayed modest shifts in dose-effect curves between WT and HET mice for the synthetic cannabinoids tested, whereas THC did not elicit CB_1-mediated reductions in body temperature in HET mice. The observed distribution of efficacy was consistent predicted *in vitro* results (A-834,735D = WIN55,212-2 ≥ CP55,940 ≥ JWH-073 ≥ CP,47,497 > THC), though only CP47,497 differed significantly from the other synthetic cannabinoids. The hypothermia

measure provides a somewhat gradual separation of potency ratios with declining efficacy, and therefore, it may be useful for differentiation of agonists with moderate to low efficacy. This moderate gradation of efficacy may be due in part to the relatively moderate expression of CB_1 in brain areas reported to modulate body temperature, notably the preoptic area (POA) of the anterior hypothalamus (AH) (Rawls *et al*, 2002) and possibly the periaqueductal grey (PAG) (Lichtman *et al*, 1996). The density of CB_1 expression in these brain areas is approximately 2 pmol/mg in rats (Herkenham *et al*, 1990). Based on the observation that a 50% reduction in CB_1 expression resulted in significant rightward shifts in the dose-response curves of cannabinoids tested suggest that hypothermia is a sensitive measure to differentiate between ligands of varying efficacy. These brain regions may also dynamically interact with one another as there are descending neuronal projections from the AH to the PAG and ascending projections in the other direction, though the interplay between these structures in regards to thermoregulation in the context of cannabinoids remains to be elucidated.

Ceiling effects for hypothermia measure were arbitrarily imposed based upon observations during pilot studies, in which an 8°C drop in body temperature was observed to coincide with maximum effects in the catalepsy and antinociception. Maximum hypothermia values actually observed sometimes reached -12°C for individual animals, though mean values at maximum doses tended to not exceed -10°C from baseline across drugs. It is possible an adjustment of the ceiling to -10°C or -12°C may reveal apparent differences in E_{max} between ligands that vary in efficacy, though this may preclude calculation of potency ratios in cases of moderate- to low- efficacy ligands, for which the calculated maximum percent effect does not sufficiently sample the linear portion of the dose-effect relationship.

The catalepsy measure was the most resistant to a 50% reduction in CB_1 expression and did not display potency ratios that diverged appreciably from a ratio of 1 for all drugs tested. Even THC, which is generally regarded as a very low-efficacy cannabinoid, displayed a very small shift in the dose-effect curve between WT and HET mice. The experimenter-imposed ceiling, underlying receptor expression, and multiple neural circuits mediating motor control may contribute to this apparent lack of sensitivity to reductions in CB_1 receptor population. Catalepsy is described as a fixed, rigid posture that is measured in seconds of immobility in either the ring or bar test. Here, we measured the latency of a given mouse to remove its forepaws from the elevated bar four times. For these experiments, a 60 second window was used to quantify this response to cannabinoids, so an ED_{50} represents the effective dose to produce 30 seconds of immobility. The actual duration of catalepsy many last for many minutes beyond the initial observation period, and as a result the maximum effect engendered by administration of each agonist to produce immobility was not measured. Practical considerations prevent the prolonged study of catalepsy in the context of the triad but it should be noted that the "maximum" effect reported here does not reflect a natural resolution of the cataleptic response but rather an experimenter imposed limit. Regardless, the potency ratios between WT and HET mice are the primary endpoint of interest and the small shifts elicited likely speak to the underlying receptor expression and/or neurobiology of the behavior.

The CB_1 receptor is expressed at very high densities in striatal regions of the midbrain in the rat (Herkenham *et al*, 1990), and expression in WT (3.33 ± 0.13 pmol/mg) and HET (1.89 ± 0.14 pmol/mg) mice are comparably high (Selley *et al*, 2001). Additionally, the receptor reserve to produce catalepsy is quite high as only a small fraction of receptor must be occupied to produce immobility (Dhawan *et al*, 2006). Thus, even under condition where 50% of the

available receptor pool is eliminated, the number of receptors needed to achieve a maximum effect remains well above what would be necessary to effect a large shift in potency. However, this apparent large receptor reserve does not entirely account for the differences among the ligands in HET mice. These mice showed no decreases in potency as measured by overlapping confidence limits for ED_{50} estimations. The rank order of the shifts in potency measured (JWH-073 ≥ A-834,735D ≥ CP47,497 ≥ CP55,940 > WIN55,212-2 ≥ THC) are also intriguing as they do not align with the predicted order (A-834,735D ≥ WIN55,212-2 > CP55,940 > JWH-073 > CP47,497 > THC). Although variability must remain a consideration, this may reflect differences in signaling events that are sufficient to produce catalepsy and are differentially activated by these compounds.

Cannabinoid signaling in the striatum and output neurons is a complicated web of signaling events that involves subregions such as the caudate-putamen (CP), the internal and external globus pallidus (GPi and GPe), entopeduncular nucleus (EP), and the substantia nigra (SN). The CP receives excitatory input from the neocortex and sends GABAergic projections to the GPi, GPe, EP, and SN. From there, dopaminergic neurons from the SN project back to the CP, while GABAergic neurons from the SN as well as the GPi project to the thalamus, which then projects back to the neocortex. In addition, the GPe and the SN send GABAergic projections to the subthalamic nucleus, which contains excitatory inputs back to the GPe, GPi, SN, and and EP. CB_1 expression is most abundant on excitatory glutamatergic neurons and inhibitory GABAergic neurons, and as a result there are many levels of regulation of synaptic transmission exerted by cannabinoids in this neuronal network. Microinjections of a CB_1 agonist into the GP suppressed locomotor activity, whereas microinjections into the SN stimulated locomotor activity (Sañudo-Peña *et al*, 1999), and systemic injections seem to produce biphasic

stimulation at low doses and stark locomotor suppression and catalepsy at doses exceeding 2.5 mg/kg (Sañudo-Peña *et al*, 2000). The exact relationship between the neurocircuitry in the striatal regions and the resulting behavior remains complex and poorly understood. However, systemic injections that produce global activation of CB_1 receptors seem to produce primarily inhibition of locomotor activity.

. Although the neurocircuitry is quite complex, CB_1 receptor expression in these regions remains quite high even in HET mice relative to other brain regions. Thus doses required to produce catalepsy would be expected to be similar in WT and HET mice, even in the case of a low efficacy agonist such as THC. Although the other agonists did not display the expected pattern of efficacy distribution, THC did elicit the largest (~2 fold) shift, consistent with the hypothesis.

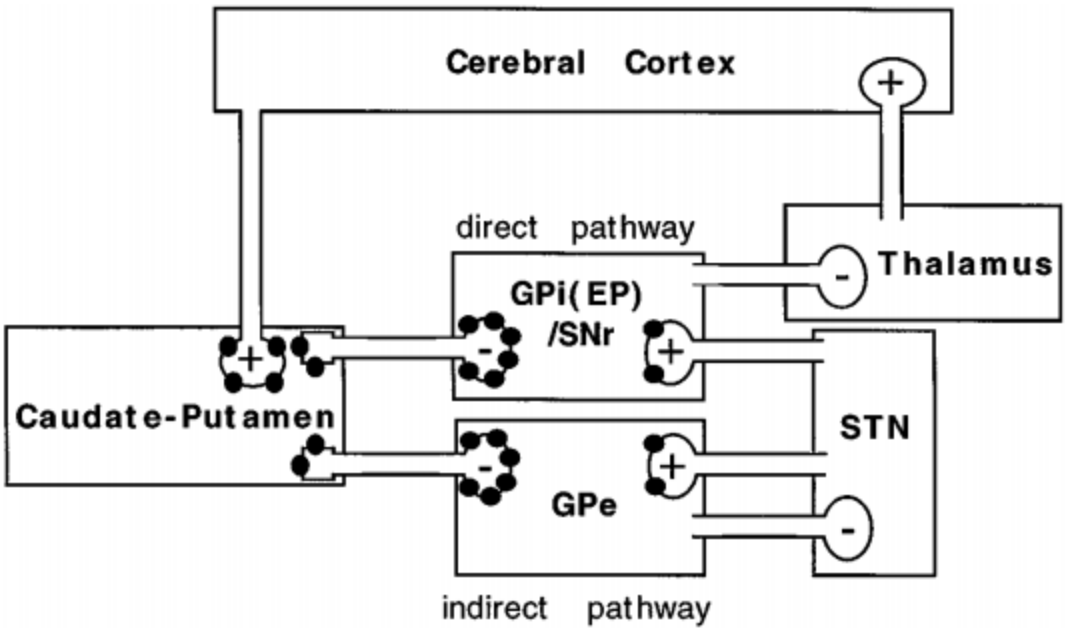

Figure 35. Adapted from Breivogel and Childers, 1998. This schematic displays the neuroanatomical projections among striatal structures. CB_1 receptors are highly expressed throughout striatal structures on GABAergic and glutamatergic neuron terminals, represented by + and - symbols.

4.4 Potential limitations of the of the *in vivo* CB_1 efficacy determination model

The cumulative dosing employed possesses limitations relative to dosing with separate groups of subjects (as discussed in the Introduction). Tachyphylaxis could certainly exacerbate potency differences, especially for low efficacy drugs, and variance in the pharmacokinetics of the test drugs could result in different optimal pretreatment times. Regardless, the half-lives of cannabinoids are in general quite long, and time-course studies revealed a long duration of action of all drugs tested using relatively high doses. Additionally, in the case of THC, cumulative dosing yields relatively similar brain levels as compared to a bolus dose 20 minutes prior to harvest (Falenski *et al*, 2010). That said, the pharmacokinetic and pharmacodynamic information regarding many compounds utilized in these studies is not well explored, and will not be examined for clandestinely synthesized SCs in the future. Generally, the half-lives of cannabinoids tend to be quite long, often on the order of hours, thus cumulative dosing is often applicable. However, there may be SCs which possess considerably shorter duration of action; therefore, rigorous assessment of peak effects along with adsorption, distribution, metabolism, and excretion will likely be necessary to further validate the model. This is especially true for CB_1 HET mice, given the paucity of information regarding specific cannabinoid actions in that context.

The *in vivo* determination of efficacy at the CB_1 receptor under these conditions is not able to differentiate between downstream signaling events, but rather, each dependent measure reflects the a function of all of the relevant signal transduction and associated biochemical events that occur to elicit a response. For instance, if interplay among multiple CB_1-mediated pathways (e.g. Gα-adenylyl cyclase-PKA, Gβγ-GIRK channels, BARR2-ERK1/2) ultimately determines antinociception in the tail flick procedure, agonist-stimulated [^{35}S]GTPγS binding alone will only

account for part of the biochemical signaling. The drugs chosen for these experiments span the efficacy continuum, although only one biochemical correlate of functional CB_1 (agonist-stimulated [^{35}S]GTPγS binding) activity was selected as a correlate. This decision was largely due to the relative abundance of knowledge regarding this signal transduction pathway in *ex vivo* preparations including acute activation and diminished activation after rats or mice were made tolerant. These data correlate well with *in vivo* effects, though given recent findings, it is unlikely these cannabinoids act solely via the canonical G-protein pathway. Whether the GDP-GTPγS exchange accurately accounts for the *in vivo* effects could be investigated more thoroughly in theory, although the relative paucity of highly biased, bioavailable CB_1 ligands precludes this sort of investigation at this time.

Although both sexes were used in all cumulative dosing triad experiments, sex-related differences among the ligands were sparse and did not conform to a discernable pattern (Table 8). Although the total number of mice used for each experiment (n=7-10 per genotype) was sufficient to detect meaningful differences among genotypes, an even split between sexes resulted in half that number when separated for analysis of sex effects (n=3-5). As a result, these comparisons were likely underpowered. Thus any significant differences detected between sexes, among genotypes, may not be meaningful. Future studies could focus on ascertaining the veracity of these sex differences, both by increasing the sample size as well as monitoring estrous cycles in females. Gonadectomies could also provide a useful method to interrogate sex hormone effects of various SCs tested should sex differences be detected.

THC	WT	HET	KO
Catalepsy	ns	ns	ns
Hypothermia	ns	ns	ns
Antinociception	ns	ns	ns

CP47,497	WT	HET	KO
Catalepsy	ns	30	ns
Hypothermia	ns	ns	ns
Antinociception	ns	100	ns

JWH-073	WT	HET	KO
Catalepsy	ns	ns	ns
Hypothermia	ns	ns	ns
Antinociception	ns	ns	ns

CP55,940	WT	HET	KO
Catalepsy	0.3	ns	1, 3
Hypothermia	ns	ns	VEH
Antinociception	ns	ns	ns

WIN55,212-2	WT	HET	KO
Catalepsy	ns	ns	ns
Hypothermia	ns	ns	ns
Antinociception	ns	ns	ns

A-834,735D	WT	HET	KO
Catalepsy	1	ns	ns
Hypothermia	ns	ns	ns
Antinociception	ns	ns	ns

Table 8. Two-way ANOVAs followed by Holm-Sidak post hoc analysis were conducted between males and females in WT, HET, and KO mice for the catalepsy, hypothermia, and antinociception measures. Significant differences were found for CP47,497, CP55,940, and A-834,735D, but not for THC, JWH-073, and WIN 55,212-2. Group sizes were small (n=3-5), thus Type I and Type II errors could not be ruled out in each case. NS indicates no significant ANOVA, all other symbols indicate specific doses for which significant sex differences were detected.

4.5 Correlations of agonist-stimulated [^{35}S]GTPγS binding with *in vivo* data: the potential for alternative pathways

The underlying purpose of GTPγS binding experiments was to discover the degree to which G-protein activity correlated with each of the triad measures. Especially when considered in the context of the agonist-antagonist interaction studies, these correlations would help reveal whether the potency shift observed between WT and HET mice *in vivo* correlated with actual differences in E_{max} measured *in vitro*. For *in vivo* data, dose ratios were calculated by dividing the WT ED_{50} by the HET ED_{50} for each triad measure and then plotted against the WT E_{max} from *in vitro* experiments. Correlations emerged for the hypothermia and antinociception measures under low receptor density conditions, suggesting G-protein activation as the primary signal transduction pathway for CB_1-mediated hypothermia and antinociception. Additionally, losses in potency in HET mice are related to reductions in functional activity at CB_1 *in vitro*.

There are numerous alternate pathways documented in addition to $G\alpha_{i/o}$ inhibition of adenylyl cyclase activity. After dissociation of the α subunit from the G-protein heterotrimer, the βγ dimer can interact with GIRK channels (Ho *et al*, 1999), and BARR1/2 recruitment is also possible, which can interfere with G-protein coupling (Breivogel *et al*, 2013), spur internalization (Daigle *et al*, 2008b), or recruit other pathways such as ERK1/2 (Flores-Otero *et al*, 2014). The results of Flores-Otero and colleagues are particularly intriguing as they suggest lengthy exposure to cannabinoid agonists shift signal transduction away from G-proteins and towards BARR2-mediated ERK1/2 phosphorylation. Utilizing radioligand binding techniques with high concentrations of $MgCl_2$ greatly extends the length of time that GTPγS remains bound to the G-proteins, which likely does not reflect the physiological equilibrium between GDP and GTP which occurs *in vivo*, and more importantly, would not capture this shift. This has the

additional consequence in the case of cumulative dosing, where prolonged exposure to the drug could result in measurement of multiple pathways at once, each dose possessing its own independent time courses to shift the equilibrium.

4.6 Lack of acute abuse-related facilitation or withdrawal effects in ICSS by CP55,940

Testing with the synthetic cannabinoid CP55,940 in the ICSS assay revealed no evidence for facilitation at any dose tested. Two high doses were tested in time course studies and revealed exclusively rate-suppressing effects for the duration of the testing period. Pretreatment with rimonabant reversed the rate-suppressing effects, indicating a CB_1-mediated mechanism of action. All of these results point to a lack of abuse liability for CP55,940 that is consistent with other reports for the same drug and other synthetic cannabinoids (Arnold *et al*, 2001; Vlachou *et al*, 2004). To date, only THC over a low and narrow dose range has displayed facilitating effects in ICSS. Species and/or strain differences may play a role as facilitation has only been shown in Lewis and Sprague-Dawley rats (Gardner *et al*, 1988; Katsidoni *et al*, 2013).

Given the abundance of cannabinoid abuse in humans, these results remain perplexing. It may be that ICSS is not well-suited to measure the abuse-related effects of THC and other cannabinoids. Another option is related to the relatively small facilitation of ICSS observed after dosing with cannabinoids. The ratio of signal to noise may preempt the ability to measure the abuse related effects. As discussed in the introduction, other assays of abuse liability also display mixed results when testing for abuse liability, so perhaps this is not surprising. Although humans, rodents, and non-human primates possess similar reward circuitry, there may be an aspect of the human experience of marijuana use that is not readily measured in preclinical assays. Thus, the majority of experiments using conventional preclinical assays of abuse potential fail to capture the apparent abuse-related effects that relate to human use.

4.7 Tolerance, but not dependence, following repeated administration of CP55,940 in ICSS

ICSS has been previously employed to measure tolerance and dependence for opioids (Altarifi and Negus, 2011), cocaine (Stoker and Markou, 2011), and nicotine (Stoker *et al*, 2012). This type of assay has also been performed following 22 days of administration of WIN55,212-2, although no evidence for tolerance or withdrawal related changes in rates of responding were detected (Mavrikaki *et al*, 2010). Conversely, rats repeatedly dosed with THC displayed tolerance to the rate-decreasing effects, though they did not exhibit rate-increasing effects at any dose nor evidence or withdrawal and/or dependence (Kwilasz and Negus, 2012). These results are interesting in the context of the results reported here with CP55,940 in which partial tolerance to the rate-decreasing effects occurred. In our studies, WIN55,212-2 maintained most of its efficacy to elicit triad effects, while CP55,940 produced slightly larger losses in potency in CB_1 HET mice implying that WIN55,212-2 is more efficacious in the whole animal. Given that both Mavrikaki et al. and our ICSS experiments utilized fixed doses, it is possible compensatory mechanisms would reduce the receptor population and the higher efficacy ligand could still elicit rate-decreasing effects of the same magnitude, whereas the somewhat lower efficacy cannabinoid would display some tolerance. Varying the dose of THC during repeated administration does result in differing degrees of tolerance to rate-decreasing effects, though no signs of dependence were unmasked similar to our results (Kwilasz and Negus, 2012).Repeated administration of a single dose of an agonist to produce tolerance does not, however, provide any information as to the loss of potency. In each case described above, cumulative dose-effect experiments before and after the repeated treatment with the selected agonist would have been a more effective approach. Still more effective would have been to vary the efficacy of the drug

used for the preceding and flanking potency assessments as has been done with drug discrimination (Hruba *et al*, 2012), or to vary the efficacy of the tolerance-inducing agents. An expanded approach might include varying the dose of the toleragen at ED_{16}, ED_{50}, and ED_{84} values to assess the dose-response relationship to induce tolerance. Regardless, efficacy might be an important determinant in the tolerance to the rate-decreasing effects of cannabinoids, but the abuse-related rate-increasing effects seem to only be detectable utilizing the low-efficacy ligand THC, and even then rather unreliably.

To test for dependence, both spontaneous and precipitated withdrawal experiments were performed following the same dosing regimen (0.3 mg/kg CP55,940, i.p., for seven days). In both cases, no disruptions in baseline responding following cessation of testing were observed, and rimonabant (10 mg/kg, i.p.) produced no rate-decreasing effects alone or after repeated dosing with CP55,940. This result is supported in the literature given the relative paucity of studies reporting significant spontaneous signs of withdrawal. The lack of precipitated withdrawal may also be related to CP55,940's ability or lack thereof to produce compensatory changes in the relevant underlying neural substrates mediating changes in ICSS during withdrawal. It may simply be that receptor occupancy was not high enough for the potent and efficacious ligand to engender sensitivity to rimonabant. To test this hypothesis, a higher dose should be used, though practical considerations must be made in the case of cannabinoids. High doses of cannabinoids for prolonged periods of time may elicit handling-induced seizures, which is consistent with CB_1 hypoactivity (von Rüden *et al*, 2015). Although not investigated, electrical brain stimulation poses a potential risk to animals with already increased susceptibility to seizures, which may impede the feasibility of these studies. Day to day rate-decreasing effects are also possible given the long half-life and duration of action of many cannabinoids which may

also confound sensitivity to tolerance. If none of these limitations are manifested, however, the relationship of efficacy, tolerance, and dependence for the CB_1 receptor may be elucidated.

4.8 Overall Conclusions

In this thesis, I have characterized the relationship of CB_1 ligands to cannabimimetic effects as measured by the cannabinoid triad (catalepsy, hypothermia, and antinociception), agonist-stimulated [^{35}S]GTPγS experiments, and abuse-related effects as measured by ICSS. The cannabinoid triad represents a novel approach of assessing *in vivo* efficacy of putative cannabinoids, endocannabinoids, and potential therapeutics such as anabolic or catabolic enzyme inhibitors. The cumulative dosing triad in CB_1 transgenic mice offers many advantages over other means of assessing *in vivo* efficacy. This assay is quick, robust, and experimental parameters may be easily modified and optimized to ascertain differences along the efficacy continuum. Additionally, emerging pharmacological tools may aid in the investigation of downstream mechanisms as they relate to CB_1 receptor density. The effect of a noncompetitive CB_1 antagonist could be assessed in WT mice to interrogate receptor reserve for agonists on different CB_1-dependent endpoints, or ligands which possess extreme bias could assess the relative contribution of various pathways to an *in vivo* endpoint. Importantly, each endpoint is differentially sensitive to reductions in receptor population providing insights into the underlying neurobiology of the endocannabinoid system. Both hypothermia and antinociception appear to be reliable measure of CB1-mediated G-protein activation *in vivo*, while catalepsy appears to be a reliable control for cannabimimetic activity in cases where it has been lost in the other two measures. Although antinociception and hypothermia are highly correlated with G-protein activation, multiple CB_1-mediated pathways may contribute to *in vivo* endpoints, which may have implications for therapeutic application of biased ligands.

In vitro studies utilizing spinal cord and cerebellum tissue from CB_1 WT, HET, and KO mice demonstrate theoretically anticipated reductions in magnitude of functional activity concordant with reductions in receptor density. We have also demonstrated for the first time a dose-effect relationship in CB_1 KO tissue with the high efficacy ligand WIN55,212-2. Although non-CB_1 stimulation has been previous reported, this stimulation has not thus far been assessed in a thorough manner during efficacy determination experiments. These results support the notion that selectivity of ligands remains important when assessing *in vitro* versus *in vivo* effects. Additionally, five of the six ligands tested here displayed similar efficacy in both high and low receptor conditions, suggesting that assay conditions could be further optimized to differentiate ligands of very high efficacy. One way to accomplish this would be to adjust the GDP/GTPγS ratio by addition of either reagent. The presented conditions did not readily differentiate between A-834,735D and CP55,940 though the expectation based upon preliminary results suggested A-834,735D might have been up to twice as efficacious to stimulate binding. Increasing the GDP concentration should elevate E_{max} values for most ligands tested, with the highest efficacy compounds displaying the largest increases (Savinainen *et al*, 2001).

Though evidence for withdrawal was not detected in ICSS following repeated treatment with CP55,940, this is the first study of its kind to assess withdrawal-related effects on reward processes in ICSS for cannabinoids and it serves to expand knowledge related to the topic. It may be that ICSS is not sensitive to detect cannabinoid withdrawal. Overall, synthetic cannabinoids are generally more potent and efficacious than THC both *in vivo* and *in vitro*. Thus they carry an increased element of risk when used by humans, especially those users that may already be tolerant.

4.9 Future directions

Though the cumulative dosing procedure was used mainly to look at the relationship between efficacy and potency shifts in CB_1 WT and HET mice, this model is quite amenable to examining efficacy under a variety of conditions. Exogenous cannabinoids can vary greatly in efficacy and so too can endogenous cannabinoids. Two primary endocannabinoid have been identified: anandamide (AEA) and 2-arachinodonoylglycerol (2-AG). In agonist-stimulated [^{35}S]GTPγS experiments, AEA has higher efficacy than THC but does not achieve the same level of activation as synthetic cannabinoids such as HU-210 and CP55,940 (Burkey *et al*, 1997a). 2-AG is much higher in efficacy than even some synthetic cannabinoids, especially under conditions of very high GDP concentrations (Savinainen *et al*, 2001). Exogenous administration of AEA and 2-AG does not typically produce cannabimimetic effects as they are rapidly catabolized by fatty acid amide hydrolase (FAAH) (Giang and Cravatt, 1997) and monoacylglycerol lipase (MAGL) (Dinh *et al*, 2002), respectively. The advent of selective pharmacological inhibitors of both FAAH (Ahn *et al*, 2009) and MAGL (Niphakis *et al*, 2013) will afford the ability to assess the *in vivo* efficacy of AEA and 2-AG in WT and HET mice. Pretreatment with an enzyme inhibitor will allow the accumulation of exogenously administered endocannabinoids, and the transgenic triad model can then be used to provide information about the functional *in vivo* activity of both AEA, 2-AG, as well as other endocannabinoids, such as noladin ether (Hanus *et al*, 2001), or hemopressin (Heimann *et al*, 2007). Importantly, CB_1 KO mice distinguish between CB_1 and non-CB_1 actions of these and other ligands.

With the ongoing discovery of clandestinely synthesized cannabinoids, a need exists to rapidly assess these compounds for their *in vitro* and *in vivo* potency and efficacy. The procedures outlined here provide a systematic, high throughput approach to screen the huge variety of novel structures available. A library of compounds already exists to conduct structure

activity relationships for the novel backbone structures being produced. Many changes in structure are small, such as fluorination or chlorination, which result in a decrease in susceptibility to be detected and to evade scheduling. Interestingly this has resulted in SAR relationships to reduce detection, but the pharmacological implications of these changes have not yet been investigated. For instance, UR-144, XLR-11, and A-834,735 are structurally related CB_1 ligands produced by Abbott Laboratories, and each possesses a heat degradant in which the tetramethylcyclopropyl ring opens. This change renders them much more efficacious to stimulate GTPγS binding, but this does not capture the entire biochemical chain of events from receptor binding to behavior that would be assessed in the CB_1 transgenic triad. It is unknown why this change results in such a large increase in efficacy, but the degree to which it relates to efficacy for a variety of biochemical assays would be interesting to study alongside the triad. Many synthetic cannabinoids agonists have nanomolar affinity for CB_2 as well as CB_1, and as a result a similar approach using CB_2 transgenic mice (Buckley *et al*, 2000) may also be used for CB_2 relevant endpoints including inflammatory or neuropathic pain and the loss of potency in CB_2 HET mice. The feasibility of cumulative dosing is an empirical question that can be answered systematically to determine the effect of repeated testing and repeated injections versus cumulative treatment with a potentially therapeutic ligand.

As discussed above, ICSS might not be well-suited to measure cannabimimetic abuse-related effects. CPP and SA are likely not good candidates either, but the drug discrimination procedure provides an alternative option. While not strictly an abuse liability assay, cannabimimetic agents such as nabilone (Lile *et al*, 2011) substitute for THC in humans, and many cannabimimetic agents display substitution for THC (Wiley *et al*, 2014, 2015), and vice versa (Järbe *et al*, 2012, 2014). Importantly, this substitution occurs in a CB_1-dependent manner

for humans (Huestis *et al*, 2001), non-human primates (McMahon, 2006), and rodents (Järbe *et al*, 2011). Therefore, drug discrimination represents one of the better options to assess the subjective effects of putative cannabinoids and by proxy their abuse liability. Although CB_1 KO mice would be unlikely to acquire a cannabimimetic agent as a discriminative stimulus, CB_1 WT and HET mice likely would providing the unique opportunity to assess the effect of receptor density on the discriminative properties of cannabimimetic agents. The intensity of the CB_1 stimulus may also be controlled by varying the training dose of the cannabimimetic agent and looking at differences in potency to substitute for the training drug in CB_1 WT and HET mice. Utilizing a very high efficacy CB_1 ligand such as A-834,735D, one could fade the training dose of the test drug, and at each level of the training drug a selection of agonists which vary in efficacy could be assessed. The hypothesis that very high efficacy drugs would maintain their potency better than low-efficacy drugs at low training is testable, and results would permit inferences into the mechanisms underlying substitution. Consistent with the anatomical distribution of CB_1 expression, generalization from cannabimimetic agents appears to be a centrally mediated phenomenon (Järbe *et al*, 2011), and as a result the cannabinoid abuse liability of these agents may be inferred. Another alternative approach would be to use THC as the discriminative stimulus, providing a low efficacy ligand to fade, and assess the maintenance of potency by high efficacy cannabinoid ligands in HET mice.

The effects of SCs upon dependence-related phenomenon are not well-characterized. One approach to explore this aspect of their pharmacology is to conduct rimonabant-precipitated withdrawal studies and vary ligands based upon efficacy. Receptor occupancy seems to drive tolerance in the case of opioids (Pawar *et al*, 2007), though similar studies have not yet been conducted for cannabinoids, and the relationship between efficacy and the severity of withdrawal

has not yet been explored. Utilizing the cumulative dosing triad as a guide for efficacy and potency determinations, one could compare the impact of repeated administration of these drugs on adaptive changes at the CB_1 receptor. From there, the dose-response relationship of rimonabant to precipitate spontaneous signs of cannabinoid withdrawal (e.g., paw flutters and head shakes) may reveal differences among the agonists differing in efficacy. It remains unknown whether repeated administration of highly efficacious SCs would result in a greater magnitude of observed withdrawal signs. In this design, the use of CB_1 KO mice will be critical to control for potential off-target effects that might occur with high doses of SCs given repeatedly. Other aspects of withdrawal could be captured with measures of anxiety-like phenotypes (e.g. marble burying or elevated plus maze) or assays of learning and/or memory (e.g. Morris water maze or object recognition tasks).

SC will likely continue to be a public health problem for years to come, and so long as clandestine chemists synthesize novel cannabimimetic agents there will be a need to assess both their basic pharmacological properties as well as potential abuse-related effects. Though overall use seems to be declining, severe health complications associated with SC abuse continue to arise (Debruyne and Le Boisselier, 2015). Here, we utilized CB_1 transgenic mice to elucidate differences in efficacy in an *in vivo* model of cannabimimetic activity, while utilizing ICSS as an assay of abuse liability. Overall, this *in vivo* approach effectively stratified agonists of varying CB_1 efficacy that showed a strong positive relationship with efficacy in the *in vitro* model of cannabimimetic activity, and would have excellent utility to assess CB_1 efficacy of emerging SCs, endogenous cannabinoids, and potential cannabinoid-based medications.

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Travis Grim, B.S.
Email: grimtw@vcu.edu
Phone: 804-334-7698
Pharmacology and Toxicology
Virginia Commonwealth University

Education

Ph.D. candidate at Virginia Commonwealth University

B.S. Psychology 2009, Virginia Tech

Publications

Grim TW, Morales AJ, Wiley JL, Thomas BF, Negus SS, Lichtman AH. Estimation of apparent pA2 and pKB values of rimonabant in the cumulative dosing cannabinoid triad. Journal of Pharmacology and Experimental Therapeutics. (*in preparation*)

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